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BIOSENSORS FOR DETECTING MACROMOLECULES AND OTHER ANALYTES

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority from PCT Application No. PCT/US04/41315 filed on December 11, 2004, which claims priority from U.S. Provisional Application Serial No. 60/529,076 filed on December 12, 2003, which are hereby incorporated by reference in their entirety.

GOVERNMENTAL RIGHTS IN INVENTION

[002] This work was supported by the U.S. Department of Health and Human Services/ National Institutes of Health grant number CA94356. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

[003] The invention relates to kits, molecular biosensors, and methods for detecting several types of target molecules, such as polypeptide, analyte, macromolecular complex, or combination thereof. The invention also relates to biomedical research tools and diagnostic kits.

BACKGROUND OF THE INVENTION

[004] The detection, identification and quantification of specific molecules in our environment, food supply, water supply and biological samples (blood, cerebral spinal fluid, urine, et cetera) can be very complex, expensive and time consuming. Methods utilized for detection of these molecules include gas chromatography, mass spectroscopy, DNA

sequencing, immunoassays, cell-based assays, biomolecular blots and gels, and myriad other multi-step chemical and physical assays.

[005] There continues to be a high demand for convenient methodologies for detecting and measuring the levels of specific proteins in biological and environmental samples. Detecting and measuring levels of proteins is one of the most fundamental and most often performed methodologies in biomedical research. While antibody-based protein detection methodologies are enormously useful in research and medical diagnostics, they are not well adapted to rapid, high-throughput parallel protein detection.

[006] Previously, the inventor had developed a fluorescent sensor methodology for detecting a specific subclass of proteins, i.e., sequence-specific DNA binding protein (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176; Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; U.S. Patent No. 6,544,746 and copending patent applications number 10/062,064, PCT/US02/24822 and PCT/US03/02157, which are incorporated herein by reference). This methodology is based on splitting the DNA binding site of proteins into two DNA "half-sites." Each of the resulting "half-sites" contains a short complementary single-stranded region of the length designed to introduce some propensity for the two DNA "half-sites" to associate recreating the duplex containing the fully functional protein binding site. This propensity is designed to be low such that in the absence of the protein only a small fraction of DNA half-sites will associate. When the protein is present in the reaction mixture, it will bind only to the duplex containing fully functional binding site. This selective binding will drive association of DNA half-sites and this protein-dependent association can be used to generate a spectroscopic signal reporting the presence of the target protein. The term "molecular beacons" is used in the art to describe the above assay to emphasize that selective recognition and generation of the signal reporting the recognition

occur in this assay simultaneously. Molecular beacons for DNA binding proteins have been developed for several proteins illustrating their general applicability (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176, which is herein incorporated by reference). Their physical mechanism of action has been established and they have been also used as a platform for the assay detecting the presence of ligands binding to DNA binding proteins (Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; Knoll, E.; Heyduk, T. *Analyt. Chem.* **2004**, 76, 1156-1164; Heyduk, E.; Fei, Y.; Heyduk, T. *Combinatorial Chemistry and High-throughput Screening* **2003**, 6, 183-194, which are incorporated herein by reference.) While already very useful, this assay is limited to proteins that exhibit natural DNA binding activity.

Aptamers as "Molecular Beacons"

[007] Development of convenient, specific, sensitive high-throughput assays for detecting proteins remains an extremely important goal. Such assays find applications in research, drug discovery and medical diagnosis. Antibodies recognizing target protein are the centerpiece of the great majority of protein detection assays so far. Development of *in vitro* methods for selecting aptamers recognizing target proteins from a population of random sequence nucleic acids provided the first real alternative to antibodies. One of the potentially important advantages of aptamers is that they are made of easy to propagate and synthesize oligonucleotides. Additionally, standard nucleic acid chemistry procedures can be used to engineer aptamers to contain reporter groups such as, for example, fluorescence probes. Thus, it is no wonder that there is a significant interest to utilize aptamers in various formats of protein detection assays. One of the most promising routes is the development of aptamer-

based sensors combining recognition of the target protein with generation of optical signal reporting the presence of the protein.

[008] There are several published reports that document ingenious designs of aptamer-based "molecular beacons" which produced fluorescence signal upon binding to a specific target protein. All of these designs rely on target protein-induced conformational transition in the aptamer to generate fluorescence signal change. Yamomoto and Kumar (*Genes to Cells* **2000,** 5, 389-396) described a molecular beacon aptamer that produced an increase of fluorescence upon recognition of HIV Tat protein. Fluorescence signal was generated due to a change of proximity of a fluorophore-quencher pair resulting from Tat protein-induced transition between hairpin and duplex forms of the aptamer. Hamaguchi et al. (*Analyt. Biochem.* **2001**, 294, 126-131) described a molecular beacon aptamer that produced an increase of fluorescence upon recognition of thrombin. In the absence of the target protein, the beacon was designed to form a stem-loop structure bringing fluorophore and the quencher to close proximity. In the presence of the protein, the beacon was forced into ligand-binding conformation resulting in increased separation between the fluorophore and the quencher and therefore, increased fluorescence signal. Li et al. (*Biochem. Biophys. Res. Commun.* **2002**, 292, 31-40) described a molecular beacon aptamer that underwent a transition from loose random coil to a compact unimolecular quadruplex in the presence of a target protein. This protein-induced change in aptamer conformation resulted in a change of proximity between fluorescence probes attached to the ends of the aptamer generating a fluorescence signal change. An analogous approach was used by Fang et al. (*ChemBioChem.* **2003**, 4, 829-834) to design a molecular beacon aptamer recognizing PDGF. These examples illustrate the great potential of aptamers for designing sensors, which could transduce the presence of the protein into an optical signal.

SUMMARY OF THE INVENTION

[009] The present invention, accordingly, is directed to molecular biosensors, methods for producing molecular biosensors and methods for using molecular biosensors. In one embodiment, the molecular biosensor is monovalent. In another embodiment, the molecular biosensor is multivalent. In one alternative of this embodiment, the molecular biosensor is bivalent. In still another alternative of this embodiment, the molecular biosensor is trivalent.

[0010] Other features and aspects of the invention are described in more detail herein.

FIGURES

[0011] Fig. 1. Overall design of molecular beacons for detecting proteins. (A) Variant of the design for targets lacking natural DNA binding activity. The beacon in this case will be composed of two aptamers developed to recognize two different epitopes of the protein. (B) Variant of the design for target exhibiting natural DNA binding activity. The beacon in this case will be composed of a short double-stranded DNA fragment containing the DNA sequence corresponding to the DNA-binding site and DNA (RNA) aptamer developed to recognize a different epitope of the protein.

[0012] Fig. 2. Methods for preparing aptamers to be used in molecular biosensors. (A) Selection of an aptamer in the presence of a known aptamer construct. The in vitro evolution process is initiated with a nucleic acid construct, an aptamer construct (composed of an known aptamer (red), a linker, and a short oligonucleotide sequence (blue)), and the target (gray). The blue color bars depict complementary short oligonucleotide sequences. (B) Simultaneous selection of two aptamers that bind distinct epitopes of the same target (gray). The in vitro evolution process is initiated with two types of nucleic acid constructs (the

primer1-2 construct and the primer3-4 construct) and the target. The blue color bar depicts short complementary sequences at the end of the two types of nucleic acid constructs. (C) Alternative design for simultaneous selection of two aptamers that bind distinct epitopes of the same target (gray). An additional pair of short oligonucleotides (blue bars) connected by a flexible linker is present during the selection process. These oligonucleotides will be complementary to short oligonucleotide sequences at the end of the nucleic acid constructs (in primer 1 and primer 4). Their presence during selection will provide a bias towards selecting pairs of aptamers capable of simultaneously binding to the target. Before cloning of the selected nucleic acid constructs the pairs of selected sequences will be ligated to preserve the information regarding the preferred pairs between various selected constructs. (D) Selection of an aptamer in the presence of a known antibody construct. The in vitro evolution process is initiated with a nucleic acid construct, an antibody construct (composed of an known antibody (red), a linker, and a short oligonucleotide sequence (blue)), and the target (gray). The blue color bars depict complementary short oligonucleotide sequences. (E) Selection of an aptamer in the presence of a known double-stranded DNA construct. The in vitro evolution process is initiated with a nucleic acid construct, an aptamer construct (composed of an known double-stranded DNA sequence (red), a linker, and a short oligonucleotide sequence (blue)), and the target (gray). The blue color bars depict complementary short oligonucleotide sequences.

[0013] Fig. 3. Comparison of the design of molecular beacons for DNA binding proteins (A) and molecular beacons for detecting proteins based on aptamers directed to two different epitopes of the protein (B).

[0014] Fig. 4. Aptamer constructs containing aptamers binding thrombin at fibrinogen exosite (60-18 [29]) and at heparin exosite (G15D).

[0015] Fig. 5. Binding of fluorescein-labeled aptamers to thrombin. (A) Binding of 60-18 [29] aptamer (THR1) (50 nM) detected by fluorescence polarization; (B) Binding of G15D aptamer (THR2) (50 nM) detected by change in fluorescence intensity; (C) Quantitative equilibrium titration of fluorescein-labeled G15D aptamer (THR2) (20 nM) with thrombin. Solid line represents nonlinear fit of experimental data to an equation describing formation of 1:1 complex between the aptamer and thrombin; (D) Quantitative equilibrium titration of fluorescein-labeled G15D aptamer (THR2) (20 nM) with thrombin in the presence of ten fold excess of unlabeled 60-18 [29] aptamer (THR3). Solid line represents nonlinear fit of experimental data to an equation describing formation of 1:1 complex between the aptamer and thrombin

[0016] Fig. 6. Illustration of the competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2) for binding to thrombin. Fluorescence spectra of 50 nM fluorescein-labeled G15D (THR2) with and without thrombin in the absence of competitor (A), in the presence of 150 nM THR3 (B), in the presence of 150 nM THR4 (C), and in the presence of 150 nM THR7 (D).

[0017] Fig. 7. Summary of experiments probing competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2) for binding to thrombin. Fluorescence intensity of fluorescein-labeled G15D aptamer (THR2) (50 nM) in the absence and the presence of the competitor (250 nM) was used to determine % of THR2 bound in the presence of the competitor. Thrombin concentration was 75 nM. The values of dissociation constants shown in the figure were calculated from a separate experiment in which 200 nM fluorescein-labeled G15D aptamer (THR2), 200 nM competitor and 150 nM thrombin were used.

[0018] Fig. 8. The effect of 60-18 [29] aptamer (THR3) on the competition between fluorescein-labeled G15D aptamer (THR2) and THR5 construct for binding to thrombin. Fluorescence spectra of 200 nM fluorescein-labeled G15D (THR2) with and without thrombin (150 nM) in the absence of the competitor (A), in the presence of 1000 nM THR3 and 200 nM THR5 (B), in the presence of 1000 nM THR3 (C), and in the presence of 200 nM THR5 (D).

[0019] Fig. 9. Binding of THR7 aptamer construct to thrombin detected by gel electrophoresis mobility shift assay. Samples of 417 nM THR7 were incubated with various amounts of thrombin (0 to 833 nM) and after 15 min incubation were loaded on a native 10% polyacrylamide gel. (A) Image of the gel stained with Sybr Green. (B) Intensity of the band corresponding to THR7-thrombin complex as a function of thrombin concentration

[0020] Fig. 10. Family of bivalent thrombin aptamer constructs in which G15D and 60-18 [29] aptamers were connected to a 20 bp DNA duplex by a 9-27 nt long poly T linker.

[0021] Fig.11. Binding of thrombin to bivalent aptamer constructs (33 nM each) illustrated in Fig. 8 detected by electrophoretic mobility shift assay (EMSA). Asterisk marks the lane best illustrating preferential binding of thrombin to constructs with 27 and 17 nt poly T linker over the constructs with 9 nt poly T linker. Thrombin concentration was varied from 0 to 400 nM.

[0022] Fig. 12. Thrombin beacon design using G15D and 60-18 [29] aptamers connected to 9 bp fluorophore (or quencher)-labeled "signaling" duplex through 17 nt poly T linker. (A) Nucleotide sequence of the fluorescein-labeled G15D construct (THR9) and dabcyl-labeled 60-18 [29] construct (THR8). (B) Mechanism of signaling by thrombin beacon. (C) Fluorescence signal change detected upon addition of thrombin to the thrombin

beacon. For comparison, titration of the fluorescein-labeled G15D construct (THR9) with thrombin in the absence of dabcyl-labeled 60-18 [29] construct (THR8) is also shown (donor only curve).

[0023] Fig. 13. A thrombin beacon design. G15D and 60-18 [29] aptamers were connected to 7 bp fluorophore (or quencher)-labeled "signaling" duplex through a linker containing 5 Spacer18 units. (A) Nucleotide sequence of the fluorescein-labeled G15D construct (THR21) and dabcyl-labeled 60-18 [29] construct (THR20). X corresponds to Spacer 18 moiety. (B) Mechanism of signaling by thrombin beacon. (C) Fluorescence signal change detected upon addition of thrombin to the thrombin beacon. For comparison, titration of the fluorescein-labeled G15D construct (THR21) with thrombin in the absence of dabcyl-labeled 60-18 [29] construct (THR20) is also shown (donor only curve). Signal change (%) was calculated as 100* (Io -I)/Io where I and Io correspond to dilution-corrected fluorescence emission intensity observed in the presence and absence of a given thrombin concentration, respectively. Inset shows fluorescence emission spectra recorded at various concentrations of thrombin corresponding to data points in the main graph.

[0024] Fig. 14. Binding of thrombin to the beacon illustrated in Fig. 13 (THR20/THR21) detected by gel electrophoresis mobility shift assay. The gel was imaged for fluorescein emission (i.e. only THR21 component of the beacon is visible).

[0025] Fig. 15. (A) Sensitivity of thrombin detection at two different concentrations of the beacon. Red circles: 50 nM THR21 and 95 nM THR20. Blue circles: 5 nM THR21 and 9.5 nM THR20. (B) Specificity of the beacon for thrombin. 50 nM THR21 and 95 nM THR20 were titrated with thrombin (red circles) and trypsin (blue circles).

[0026] Fig. 16. Reversal of thrombin beacon signal by competitor aptamer constructs. Fluorescence intensity of 50 nM THR21, 95 nM THR20, and 100 nM thrombin

was measured at increasing concentrations of competitor DNA's. The data are plotted as a relative fluorescence increase with respect to a signal (Fo) of a starting beacon and thrombin mixture. Open blue squares: THR7; filled black circles: THR14/THR15; filled red squares: THR16/THR17; filled blue triangles: THR18/THR19; open magenta triangles: THR3; green filled inverted triangles: THR4; open black triangles: nonspecific single stranded DNA.

[0027] Fig. 17 The binding of aptamer constructs to thrombin. (A) Binding of G15D aptamer (THR2) (50 nM) detected by change in fluorescence intensity of 5' fluorescein moiety. Solid line represents the best fit of the experimental data to a simple 1:1 binding isotherm. (B) Binding of G15D aptamer (THR2) in the presence of 10x excess of unlabeled 60-18 [29] aptamer. Solid line represents the best fit of the experimental data to a simple 1:1 binding isotherm. (C) Summary of experiments probing competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2). Fluorescence intensity of THR2 (200 nM) was used to determine % THR2 bound in the presence of competitor (200 nM). Thrombin was 150 nM. The labels above each bar indicate relative affinity (expressed as fold increase of affinity constant) of the competitor compared to the affinity of THR2 aptamer. (D) Binding of THR7 aptamer construct to thrombin detected by gel electrophoresis mobility shift assay. Intensity of the band corresponding to THR7-thrombin complex is plotted as a function of thrombin concentration. Inset: Image of the gel stained with Sybr Green. Fluorescence change (%) was calculated as 100* (I-Io)/Io where I and Io correspond to dilution-corrected fluorescence emission intensity observed in the presence and absence of a given thrombin concentration, respectively.

[0028] Fig. 18 Variants of thrombin beacon with various combinations of donor-acceptor fluorophores. (A) fluorescein-dabcyl; (B) fluorescein-Texas Red; (C) fluorescein-Cy5, (D) Cy3-Cy5. Emission spectra of the beacon in the absence (black line) and presence

(red line) of thrombin are shown. Insets show false color images of microplate wells containing corresponding beacon and indicated concentrations of thrombin. The images were obtained on Bio-Rad Molecular Imager FX using the following excitation-emission settings: (A) 488 nm laser – 530 nm bandpass filter; (B) 488 nm laser – 640 nm bandpass filter; (C) 488 nm laser – 695 nm bandpass filter; (D) 532 nm laser – 695 nm bandpass filter. Fluorescence is in arbitrary units (corrected for instrument response) and is plotted in a linear scale.

[0029] Fig. 19 Response curves for the beacon with various combinations of donor-acceptor pairs. (A) fluorescein-dabcyl, (B) fluorescein-Texas Red, (C) Cy3-Cy5, (D) fluorescein-Cy5, (E) europium chelate-Cy5, (F) Fold signal change observed for indicated donor-acceptor pair at saturating thrombin concentration. Insets show expanded view of data points at low thrombin concentrations. In all experiments 5 nM donor-labeled and 5.5 nM acceptor-labeled aptamer constructs were used. Signal change (fold) was calculated as I/Io where I and Io correspond to dilution-corrected acceptor fluorescence emission intensity (measured with donor excitation) observed in the presence and absence of a given thrombin concentration, respectively. Buffer background was subtracted from I and Io before calculating signal change.

[0030] Fig. 20 The dependence of the sensitivity of the thrombin beacon on a donor-acceptor pair. Response of 10 nM donor-labeled and 11 nM acceptor-labeled beacon was determined at low thrombin concentrations using beacon labeled with fluorescein-dabcyl pair (triangles), fluorescein-Texas Red pair (inverted triangles), and fluorescein-Cy5 pair (circles). Averages and standard deviations of four independent experiments are shown.

[0031] Fig. 21 The reproducibility and stability of thrombin beacon. (A) Five independent determinations of beacon signal at four different thrombin concentrations were

performed. Data shown represent mean +/- standard deviation. (B) Thrombin beacon signal at four thrombin concentrations was monitored over time up to 24 hours. Data shown represent mean +/- standard deviation of 5 independent measurements. Beacon containing 5 nM fluorescein-labeled aptamer (THR21) and 5.5 nM Texas Red-labeled aptamer (THR27) was used in this experiment.

[0032] Fig. 22 shows the determination of Z'-factor for thrombin beacon. Panel in the middle of the plot shows the false color image of wells of the microplate corresponding to the experiment shown in a graph (the upper half of wells are + thrombin, the lower half of the wells is – thrombin. Beacon containing with 5 nM fluorescein-labeled aptamer (THR21) and 5.5 nM Texas Red-labeled aptamer (THR27) was used in this experiment. Signal corresponds to a ratio of acceptor to donor emission (in arbitrary units) measured with donor excitation.

[0033] Fig. 23 The detection of thrombin in complex mixtures. (A) Response of thrombin beacon at 1 nM thrombin concentration in the absence and presence of the excess of unrelated proteins. The data shown are averages and standard deviation of 4 independent experiments. (B) Detection of thrombin in HeLa extract "spiked" with various amounts of thrombin. Data shown are averages and standard deviation from 3 independent measurements. Concentrations of thrombin in cell extract were: 1.88 nM (green bars); 3.75 nM (pink bars); 7.5 nM (red bars). Signal for beacon mixture alone was ~ 25% lower then when cell extract (no thrombin added) was present (not shown) which was essentially the same as the signal observed in the presence of cell extract and specific competitor. (C) Time course of prothrombin to thrombin conversion catalyzed by Factor Xa monitored by thrombin beacon. (D) Detection of thrombin in plasma. Data shown are averages and standard deviation from 4 independent measurements. The volumes of plasma used (per 20 μl assay

mixture) were: 0.005 μl (green bars); 0.015 μl (red bars); 0.045 μl (blue bars). "Specific" refers to unlabeled thrombin aptamer competitor (THR7) whereas "nonspecific" refers to random sequence 30 nt DNA. Signal in panels A, B and D corresponds to a ratio of acceptor to donor emission measured with donor excitation. Signals were normalized to value of 1 for beacon mixture alone (panels A and D) and beacon mixture in the presence of cell extract (panel B). Panel C shows raw acceptor fluorescence intensity (with donor excitation).

[0034] Fig. 24 Various formations of molecular biosensors.

[0035] Fig. 25 The experimental demonstration of sensor design shown in Fig. 24F. (A) Principle of sensor function. (B) Increase of sensitized acceptor fluorescence upon titration of increasing concentrations of DNA binding protein to the mixture of donor and acceptor labeled sensor components.

[0036] Fig. 26 The experimental demonstration of a functioning sensor design shown in Fig. 24G. (A) Principle of sensor function. (B) Increase of sensitized acceptor fluorescence (emission spectrum labeled with "+") upon addition of single-stranded DNA containing two distinct sequence elements complementary to sensor elements to the mixture of two donor and acceptor labeled sensor components (spectrum labeled with "-").

[0037] Fig. 27 The experimental demonstration of the increased specificity of our sensor design compared to assays based on a single, target macromolecule-recognizing element.

[0038] Fig. 28 summarizes the selection of an aptamer that binds to thrombin at an epitope distinct from the binding site of the G15D aptamer. (A) An illustration of the reagents used to begin the process of selection. (B) The graph indicates the increase in thrombin binding with successive rounds of selection. (C) The sequences represent aptamers developed after 12 rounds of selection.

[0039] Fig. 29 The demonstration of the functional thrombin sensor comprising Texas Red-labeled THR27 and fluorescein-labeled THR35 or THR36 (both contain the sequence corresponding to that of clones 20, 21, 24, and 26 of Figure 5C). The fluorescence image represents the specificity of either 20nM (panel A) or 100nM (panel B) of the indicated biosensor.

[0040] Fig. 30 summarizes the simultaneous selection of two aptamers that bind to thrombin at distinct epitopes. (A) An illustration of the reagents used to begin the process of selection. (B) The graph indicates the increase in thrombin binding with successive rounds of selection. (C) The sequences represent aptamers developed after 13 rounds of selection.

[0041] Fig. 31 summarizes the selection of an aptamer that binds to CRP at an epitope distinct from the DNA-binding site. (A) An illustration of the reagents used to begin the process of selection. (B) The graph indicates the increase in thrombin binding with successive rounds of selection. (C) The sequences represent aptamers developed after 11 rounds of selection. A summary of the selection of the aptamer binding to thrombin at an epitope distinct from the binding site of the G15D aptamer.

[0042] Fig. 32 A diagram of methods for permanently linking the two aptamers recognizing two distinct epitopes of the target. (A) Two complimentary oligonucleotides are attached using linkers to each of the aptamers. These oligonucleotides are long enough (typically >15bps) to form a stable duplex permanently linking the two aptamers. (B) The two aptamers are connected directly via a linker.

[0043] Fig. 33 Example of a potential sensor design utilizing three sensing components. In this design the target is a complex of three components (light blue, dark blue, and black ovals). Each of the aptamers recognizes one of the components of the complex. Signals of different color from each of the two signaling oligonucleotide pairs could be sued

to discriminate between the entire complex containing all three components with alternative sub-complexes containing only tow of the components.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0044] The present invention is directed to a novel class of molecular biosensors. The molecular biosensors may be utilized in several different methods, such as for the detection of a target molecule. To detect a target molecule, the method typically involves target-molecule induced co-association of two epitope-binding agents that each recognize distinct epitopes on the target molecule. The epitope-binding agents each comprise complementary signaling oligonucleotides that are labeled with detection means and are attached to the epitope binding agents through a flexible linker. Co-association of the two epitope-binding agents with the target molecule results in bringing the two signaling oligonucleotides into proximity such that a detectable signal is produced. Advantageously, the molecular biosensors provide a rapid homogeneous means to detect a variety of target molecules, including but not limited to proteins, carbohydrates, macromolecules, and analytes.

(I) Molecular Biosensors

[0045] One aspect of the invention, accordingly, encompasses a molecular biosensor. Several molecular configurations of biosensors are suitable for use in the invention as illustrated by way of non limiting example in FIGs. 24 and 33. In one embodiment, the molecular biosensor will be monovalent comprising a single epitope binding agent that binds to an epitope on a target molecule. The molecular biosensor of the invention, however, is typically multivalent. It will be appreciated by a skilled artisan, depending upon

the target molecule, that the molecular biosensor may comprise from about 2 to about 5 epitope binding agents. Typically, the molecular biosensor will comprise 2 or 3 epitope binding agents and more typically, will comprise 2 epitope binding agents. In one alternative of this embodiment, therefore, the molecular biosensor will be bivalent comprising a first epitope binding agent that binds to a first epitope on a target molecule and a second epitope binding agent that binds to a second epitope on the target molecule. In yet alternative of this embodiment, the molecular biosensor will be trivalent comprising a first epitope binding agent that binds to a first epitope on a target molecule, a second epitope binding agent that binds to a second epitope on a target molecule and a third epitope binding agent that binds to a third epitope on a target molecule.

a. Bivalent Molecular Sensors

[0046] In one alternative of the invention, the molecular biosensor will be bivalent. In a typical embodiment, the bivalent construct will comprise a first epitope binding agent that binds to a first epitope on a target molecule, a first linker, a first signaling oligo, a first detection means, a second epitope binding agent that binds to a second epitope on the target molecule, a second linker, a second signaling oligo, and a second detection means.

[0047] In one preferred embodiment, the molecular biosensor comprises two nucleic acid constructs, which together have formula (I):

i. R^1-R^2-R^3-R^4; and

ii. R^5-R^6-R^7-R^8; (I)

wherein:

R^1 is an epitope binding agent that binds to a first epitope on a target molecule;

R^2 is a flexible linker attaching R^1 to R^3;

R^3 and R^7 are a pair of complementary nucleotide sequences having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^4 and R^8 together comprise a detection means such that when R^3 and R^7 associate a detectable signal is produced;

R^5 is an epitope binding agent that binds to a second epitope on the target molecule; and

R^6 is a flexible linker attaching R^5 to R^7.

[0048] As will be appreciated by those of skill in the art, the choice of epitope binding agents, R^1 and R^5, in molecular biosensors having formula (I) can and will vary depending upon the particular target molecule. By way of example, when the target molecule is a protein, R^1 and R^5 may be an aptamer, or antibody. By way of further example, when R^1 and R^5 are double stranded nucleic acid the target molecule is typically a macromolecule that binds to DNA or a DNA binding protein. In general, suitable choices for R^1 and R^5 will include two agents that each recognize distinct epitopes on the same target molecule. In certain embodiments, however, it is also envisioned that R^1 and R^5 may recognize distinct epitopes on different target molecules. Non-limiting examples of suitable epitope binding agents, depending upon the target molecule, include agents selected from the group consisting of an aptamer, an antibody, an antibody fragment, a double-stranded DNA sequence, a ligand, a ligand fragment, a receptor, a receptor fragment, a polypeptide, a peptide, a coenzyme, a coregulator, an allosteric molecule, and an ion. In an exemplary embodiment, R^1 and R^5 are each aptamers having a sequence ranging in length from about 20 to about 110 bases. In another embodiment, R^1 and R^5 are each antibodies selected from the group consisting of polyclonal antibodies, ascites, Fab fragments, Fab' fragments,

monoclonal antibodies, and humanized antibodies. In a preferred embodiment, R^1 and R^5 are each monoclonal antibodies. In an additional embodiment, R^1 and R^5 are each double stranded DNA. In a further embodiment, R^1 is a double stranded nucleic acid and R^5 is an aptamer. In an additional embodiment, R^1 is an antibody and R^5 is an aptamer. In an additional embodiment, R^1 is an antibody and R^5 is a double stranded DNA.

[0049] In an additional embodiment for molecular biosensors having formula (I), exemplary linkers, R^2 and R^6, will functionally keep R^3 and R^7 in close proximity such that when R^1 and R^5 each bind to the target molecule, R^3 and R^7 associate in a manner such that a detectable signal is produced by the detection means, R^4 and R^8. R^2 and R^6 may each be a nucleotide sequence from about 10 to about 100 nucleotides in length. In one embodiment, R^2 and R^6 are from 10 to about 25 nucleotides in length. In another embodiment, R^2 and R^6 are from about 25 to about 50 nucleotides in length. In a further embodiment, R^2 and R^6 are from about 50 to about 75 nucleotides in length. In yet another embodiment, R^2 and R^6 are from about 75 to about 100 nucleotides in length. In each embodiment, the nucleotides comprising the linkers may be any of the nucleotide bases in DNA or RNA (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA). In one embodiment R^2 and R^6 are comprised of DNA bases. In another embodiment, R^2 and R^6 are comprised of RNA bases. In yet another embodiment, R^2 and R^6 are comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. Alternatively, R^2 and R^6 may be a polymer of bifunctional chemical linkers. In one embodiment the bifunctional chemical linker is heterobifunctional. Suitable heterobifunctional chemical linkers include sulfoSMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and lc-SPDP(N-

Succinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate). In another embodiment the bifunctional chemical linker is homobifunctional. Suitable homobifunctional linkers include disuccinimidyl suberate, disuccinimidyl glutarate, and disuccinimidyl tartrate. Additional suitable linkers are illustrated in the Examples, such as the phosphoramidate form of Spacer 18 comprised of polyethylene glycol. In one embodiment, R^2 and R^6 are from 0 to about 500 angstroms in length. In another embodiment, R^2 and R^6 are from about 20 to about 400 angstroms in length. In yet another embodiment, R^2 and R^6 are from about 50 to about 250 angstroms in length.

[0050] In a further embodiment for molecular biosensors having formula (I), R^3 and R^7 are complementary nucleotide sequences having a length such that they preferably do not associate unless R^1 and R^5 bind to separate epitopes on the target molecule. When R^1 and R^5 bind to separate epitopes of the target molecule, R^3 and R^7 are brought to relative proximity resulting in an increase in their local concentration, which drives the association of R^3 and R^7. R^3 and R^7 may be from about 2 to about 20 nucleotides in length. In another embodiment, R^3 and R^7 are from about 4 to about 15 nucleotides in length. In an exemplary embodiment, R^3 and R^7 are from about 5 to about 7 nucleotides in length. In one embodiment, R^3 and R^7 have a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions, defined below. In another embodiment, R^3 and R^7 have a free energy for association from about 6.0 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions defined below. In yet another embodiment, R^3 and R^7 have a free energy for association from about 7.0 kcal/mole to 8.0 kcal/mole in the selection buffer conditions. In a preferred embodiment, R^3 and R^7 have a free energy for association of 7.5 kcal/mole in the selection buffer conditions described below. Preferably, in each embodiment R^3 and R^7 are not complementary to R^1 and R^5.

[0051] In a typical embodiment for molecular biosensors having formula (I), R^4 and R^8 may together comprise several suitable detection means such that when R^3 and R^7 associate, a detectable signal is produced. Exemplary detections means suitable for use in the molecular biosensors include FRET, fluorescence cross-correlation spectroscopy, flourescence quenching, fluorescence polarization, flow cytometry, scintillation proximity, luminescence resonance energy transfer, direct quenching, ground-state complex formation, chemiluminescence energy transfer, bioluminescence resonance energy transfer, excimer formation, colorimetric substrates detection, phosphorescence, electro-chemical changes, and redox potential changes.

[0052] In a further embodiment, the molecular biosensor will have formula (I) wherein:

R^1 is an epitope binding agent that binds to a first epitope on a target molecule and is selected from the group consisting of an aptamer, an antibody, and double stranded nucleic acid;

R^2 is a flexible linker attaching R^1 to R^3 by formation of a covalent bond with each of R^1 and R^3, wherein R^2 comprises a bifunctional chemical cross linker and is from 0 to 500 angstroms in length;

R^3 and R^7 are a pair of complementary nucleotide sequences from about 4 to about 15 nucleotides in length and having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^4 and R^8 together comprise a detection means selected from the group consisting of FRET, fluorescence cross-correlation spectroscopy, flourescence quenching, fluorescence polarization, flow cytometry, scintillation proximity, luminescence resonance energy transfer,

direct quenching, ground-state complex formation, chemiluminescence energy transfer, bioluminescence resonance energy transfer, excimer formation, colorimetric substrates detection, phosphorescence, electro-chemical changes, and redox potential changes;

R^5 is an epitope binding agent that binds to a second epitope on the target molecule and is selected from the group consisting of an aptamer, an antibody, and double stranded nucleic acid; and

R^6 is a flexible linker attaching R^5 to R^7 by formation of a covalent bond with each of R^5 and R^7, wherein R^6 comprises a bifunctional chemical cross linker and is from 0 to 500 angstroms in length.

[0053] Yet another embodiment of the invention encompasses a molecular biosensor having formula (I) wherein:

R^1 is an aptamer that binds to a first epitope on a target molecule;

R^2 is a flexible linker attaching R^1 to R^3;

R^3 and R^7 are a pair of complementary nucleotide sequences having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^4 and R^8 together comprise a detection means such that when R^3 and R^7 associate a detectable signal is produced;

R^5 is an aptamer that binds to a second epitope on the target molecule; and

R^6 is a flexible linker attaching R^5 to R^7.

[0054] A further embodiment of the invention encompasses a molecular biosensor having formula (I) wherein:

R^1 is an aptamer that binds to a first epitope on a target molecule;

R^2 is a flexible linker attaching R^1 to R^3 by formation of a covalent bond with each of R^1 and R^3, wherein R^2 comprises a bifunctional chemical cross linker and is from 0 to 500 angstroms in length;

R^3 and R^7 are a pair of complementary nucleotide sequence from about 4 to about 15 nucleotides in length and having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^4 and R^8 together comprise a detection means selected from the group consisting of FRET, fluorescence cross-correlation spectroscopy, flourescence quenching, fluorescence polarization, flow cytometry, scintillation proximity, luminescence resonance energy transfer, direct quenching, ground-state complex formation, chemiluminescence energy transfer, bioluminescence resonance energy transfer, excimer formation, colorimetric substrates detection, phosphorescence, electro-chemical changes, and redox potential changes;

R^5 is an aptamer that binds to a second epitope on the target molecule; and

R^6 is a flexible linker attaching R^5 to R^7 by formation of a covalent bond with each of R^5 and R^7, wherein R^6 comprises a bifunctional chemical cross linker and is from 0 to 500 angstroms in length.

[0055] In each of the foregoing embodiments for molecular biosensors having formula (I), the first nucleic acid construct, R^1-R^2-R^3-R^4, and the second nucleic acid construct, R^5-R^6-R^7-R^8, may optionally be attached to each other by a linker R^{LA} to create tight binding bivalent ligands. Typically, the attachment is by covalent bond formation. Alternatively, the attachment may be by non covalent bond formation. In one embodiment, R^{LA} attaches R^1 of the first nucleic acid construct to R^5 of the second nucleic acid construct to form a molecule comprising:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4$$
$$R^{LA} \;\|$$
$$R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

[0056] In a further embodiment, R^{LA} attaches R^2 of the first nucleic acid construct to R^6 of the second nucleic acid construct to form a molecule comprising:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4$$
$$R^{LA} \;\|$$
$$R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

[0057] In yet another embodiment, R^{LA} attaches R^3 of the first nucleic acid construct to R^7 of the second nucleic acid construct to form a molecule comprising:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4$$
$$R^{LA} \;\|$$
$$R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

[0058] Generally speaking, R^{LA} may be a nucleotide sequence from about 10 to about 100 nucleotides in length. The nucleotides comprising R^{LA} may be any of the nucleotide bases in DNA or RNA (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA). In one embodiment, R^{LA} is comprised of DNA bases. In another embodiment, R^{LA} is comprised of RNA bases. In yet another embodiment, R^{LA} is comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. Alternatively, R^{LA} may be a polymer of bifunctional chemical linkers. In one embodiment the bifunctional chemical linker is heterobifunctional. Suitable heterobifunctional chemical linkers include

sulfoSMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and lc-SPDP(N-Succinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate). In another embodiment the bifunctional chemical linker is homobifunctional. Suitable homobifunctional linkers include disuccinimidyl suberate, disuccinimidyl glutarate, and disuccinimidyl tartrate. An exemplary R^{LA} is the phosphoramidate form of Spacer 18 comprised of polyethylene glycol. In one embodiment, R^{LA} is from about 1 to about 500 angstroms in length. In another embodiment, R^{LA} is from about 20 to about 400 angstroms in length. In yet another embodiment, R^{LA} is from about 50 to about 250 angstroms in length.

Trivalent Molecular Sensors

[0059] In an additional alternative embodiment, the molecular biosensor will be trivalent. In a typical embodiment, the trivalent construct will comprise a first epitope binding agent that binds to a first epitope on a target molecule, a first linker, a first signaling oligo, a first detection means, a second epitope binding agent that binds to a second epitope on the target molecule, a second linker, a second signaling oligo, a second detection means, a third epitope binding agent that binds to a third epitope on a target molecule, a third linker, a third signaling oligo, and a third detection means.

[0060] In one preferred embodiment, the molecular biosensor comprises three nucleic acid constructs, which together have formula (II):

i. R^{15}-R^{14}-R^{13}-R^{9}-R^{10}-R^{11}-R^{12};

ii. R^{16}-R^{17}-R^{18}-R^{19}; and

iii. R^{20}-R^{21}-R^{22}-R^{23} (II)

wherein:

R^9 is an epitope binding agent that binds to a first epitope on a target molecule;

R^{10} is a flexible linker attaching R^9 to R^{11};

R^{11} and R^{22} are a first pair of complementary nucleotide sequences having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^{12} and R^{23} together comprise a detection means such that when R^{11} and R^{22} associate a detectable signal is produced;

R^{13} is a flexible linker attaching R^9 to R^{14};

R^{14} and R^{18} are a second pair of complementary nucleotide sequences having a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from about 21° C to about 40° C and at a salt concentration from about 1 mM to about 100 mM;

R^{15} and R^{19} together comprise a detection means such that when R^{14} and R^{18} associate a detectable signal is produced;

R^{16} is an epitope binding agent that binds to a second epitope on a target molecule;

R^{17} is a flexible linker attaching R^{16} to R^{18};

R^{20} is an epitope binding agent that binds to a third epitope on a target molecule; and

R^{21} is a flexible linker attaching R^{20} to R^{22}.

[0061] The choice of epitope binding agents, R^9, R^{16} and R^{20}, in molecular biosensors having formula (II) can and will vary depending upon the particular target molecule. Generally speaking, suitable choices for R^9, R^{16} and R^{20} will include three agents that each recognize distinct epitopes on the same target molecule or on different target molecules. Non-limiting examples of suitable epitope binding agents, depending upon the target molecule(s), include agents selected from the group consisting of an aptamer, an antibody, an antibody fragment, a double-stranded DNA sequence, a ligand, a ligand fragment, a receptor, a receptor fragment, a polypeptide, a peptide, a coenzyme, a coregulator, an allosteric molecule, and an ion. In an exemplary embodiment, R^9, R^{16} and R^{20}

are each aptamers having a sequence ranging in length from about 20 to about 110 nucleotide bases.

[0062] In an additional embodiment for molecular biosensors having formula (II), exemplary linkers, R^{10} and R^{21}, will functionally keep R^{11} and R^{22} in close proximity such that when R^9 and R^{20} each bind to the target molecule(s), R^{11} and R^{22} associate in a manner such that a detectable signal is produced by the detection means, R^{12} and R^{23}. In addition, exemplary linkers, R^{13} and R^{17}, will functionally keep R^{14} and R^{18} in close proximity such that when R^9 and R^{16} each bind to the target molecule(s), R^{14} and R^{18} associate in a manner such that a detectable signal is produced by the detection means, R^{15} and R^{19}. In one embodiment, the linkers utilized in molecular biosensors having formula (II) may each be a nucleotide sequence from about 10 to about 100 nucleotides in length. In one embodiment, the linkers are from 10 to about 25 nucleotides in length. In another embodiment, the linkers are from about 25 to about 50 nucleotides in length. In a further embodiment, the linkers are from about 50 to about 75 nucleotides in length. In yet another embodiment, the linkers are from about 75 to about 100 nucleotides in length. In each embodiment, the nucleotides comprising the linkers may be any of the nucleotide bases in DNA or RNA (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA). In one embodiment, the linkers are comprised of DNA bases. In another embodiment, the linkers are comprised of RNA bases. In yet another embodiment, the linkers are comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. Alternatively, the linkers may be a polymer of bifunctional chemical linkers. In one embodiment the bifunctional chemical linker is heterobifunctional. Suitable heterobifunctional chemical linkers include sulfoSMCC

(Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and lc-SPDP(N-Succinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate). In another embodiment the bifunctional chemical linker is homobifunctional. Suitable homobifunctional linkers include disuccinimidyl suberate, disuccinimidyl glutarate, and disuccinimidyl tartrate. Additional suitable linkers are illustrated in the Examples, such as the phosphoramidate form of Spacer 18 comprised of polyethylene glycol. In one embodiment, the linkers are from 0 to about 500 angstroms in length. In another embodiment, the linkers are from about 20 to about 400 angstroms in length. In yet another embodiment, the linkers are from about 50 to about 250 angstroms in length.

[0063] In a further embodiment for molecular biosensors having formula (II), R^{11} and R^{22} are complementary nucleotide sequences having a length such that they preferably do not associate unless R^9 and R^{20} bind to separate epitopes on the target molecule(s). In addition, R^{14} and R^{18} are complementary nucleotide sequences having a length such that they preferably do not associate unless R^9 and R^{16} bind to separate epitopes on the target molecule(s). R^{11} and R^{22} and R^{14} and R^{18} may be from about 2 to about 20 nucleotides in length. In another embodiment, R^{11} and R^{22} and R^{14} and R^{18} are from about 4 to about 15 nucleotides in length. In an exemplary embodiment, R^{11} and R^{22} and R^{14} and R^{18} are from about 5 to about 7 nucleotides in length. In one embodiment, R^{11} and R^{22} and R^{14} and R^{18} have a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions, defined below. In another embodiment, R^{11} and R^{22} and R^{14} and R^{18} have a free energy for association from about 6.0 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions defined below. In yet another embodiment, R^{11} and R^{22} and R^{14} and R^{18} have a free energy for association from about 7.0 kcal/mole to 8.0 kcal/mole in the selection buffer conditions. In a preferred embodiment, R^{11}

and R^{22} and R^{14} and R^{18} have a free energy for association of 7.5 kcal/mole in the selection buffer conditions described below. Preferably, in each embodiment R^{11} and R^{22} and R^{14} and R^{18} are not complementary to any of R^{9}, R^{16} or R^{20}.

[0064] In a typical embodiment for molecular biosensors having formula (II), R^{12} and R^{23} may together comprise several suitable detection means such that when R^{11} and R^{22} associate, a detectable signal is produced. In addition, R^{15} and R^{19} may together comprise several suitable detection means such that when R^{14} and R^{18} associate, a detectable signal is produced. Exemplary detections means suitable for use in the molecular biosensors include FRET, fluorescence cross-correlation spectroscopy, flourescence quenching, fluorescence polarization, flow cytometry, scintillation proximity, luminescence resonance energy transfer, direct quenching, ground-state complex formation, chemiluminescence energy transfer, bioluminescence resonance energy transfer, excimer formation, colorimetric substrates detection, phosphorescence, electro-chemical changes, and redox potential changes.

II. Methods for Selecting Epitope Binding Agents

[0065] A further aspect of the invention provides methods for selecting epitope binding agents, and in particular aptamers for use in making any of the molecular biosensors of the present invention. Generally speaking, epitope binding agents comprising aptamers, antibodies or double stranded DNA may be purchased if commercially available or may be made in accordance with methods generally known in the art.

[0066] The invention, however, provides novel methods for simultaneously selecting two or more aptamers that each recognize distinct epitopes on a target molecule or on separate target molecules. Alternatively, the invention also provides novel methods directed to selecting at least one aptamer in the presence of an epitope binding agent

construct. The aptamer and epitope binding agent construct also each recognize distinct epitopes on a target molecule.

i. Method for Selection of an Aptamer in the Presence of an Epitope Binding Agent Construct

[0067] One aspect of the invention encompasses a method for selecting an aptamer in the presence of an epitope binding agent construct. The aptamer and epitope binding agent construct are selected so that they each bind to the same target at two distinct epitopes. Typically, the method comprises contacting a plurality of nucleic acid constructs and epitope binding agent constructs with a target molecule to form a mixture. The mixture will generally comprise complexes having target molecule bound with nucleic acid constructs and epitope binding agent constructs. According to the method, the complex is isolated from the mixture and the nucleic acid construct is purified from the complex. The aptamer selected by the method of the invention will comprise the purified nucleic acid construct.

[0068] In this method of selection, a plurality of nucleic acid constructs is utilized in the presence of the epitope binding agent construct to facilitate aptamer selection. The nucleic acid constructs comprise:

i. A-B-C-D

The epitope binding agent construct comprises:

ii. P-Q-R

wherein:

A and C are each different DNA sequences from about 10 to about 30 nucleotides in length, A and C together comprising a sequence to prime a polymerase chain reaction for amplifying the aptamer sequence;

B is a single-stranded nucleotide random sequence from about 20 to about 110 nucleotides in length that contains specific sequences binding to a first epitope of the target molecule;

D and R are a pair of complementary nucleotide sequences from about 2 to about 20 nucleotides in length, wherein D and R have a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from approximately 21° C to about 40° C and at a salt concentration of approximately 1 mM to about 100 mM;

P is an epitope binding agent that binds to a second epitope on the target molecule. The epitope binding agent will vary depending upon the embodiment, but is selected from the group comprising an aptamer, an antibody, an antibody fragment, a double-stranded DNA sequence, a ligand, a ligand fragment, a receptor, a receptor fragment, a polypeptide, a peptide, a coenzyme, a coregulator, an allosteric molecule, and an ion; and

Q is a flexible linker.

[0069] Generally speaking, A and C are each different DNA sequences ranging from about 7 to about 35 nucleotides in length and function as polymerase chain reaction primers to amplify the nucleic acid construct. In another embodiment, A and C range from about 15 to about 25 nucleotides in length. In yet another embodiment, A and C range from about 15 to about 20 nucleotides in length. In still another embodiment, A and C range from about 16 to about 18 nucleotides in length. In an exemplary embodiment, A and C are 18 nucleotides in length. Typically, A and C have an average GC content from about 53% to 63%. In another embodiment, A and C have an average GC content from about 55% to about 60%. In a preferred embodiment, A and C will have an average GC content of about 60%.

[0070] B is typically a single-stranded oligonucleotide synthesized by randomly selecting and inserting a nucleotide base (A, C, T, G in the case of DNA, or A, C, U, G in the

case of RNA) at every position of the oligonucleotide. In one embodiment, B encodes an aptamer sequence that binds to the first epitope on the target. In another embodiment B is comprised of DNA bases. In yet another embodiment, B is comprised of RNA bases. In another embodiment, B is comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. In a further embodiment, B is about 20 to 110 nucleotides in length. In another embodiment, B is from about 25 to about 75 nucleotides in length. In yet another embodiment, B is from about 30 to about 60 nucleotides in length.

[0071] In one embodiment, D and R are complementary nucleotide sequences from about 2 to about 20 nucleotides in length. In another embodiment, D and R are from about 4 to about 15 nucleotides in length. In a preferred embodiment, D and R are from about 5 to about 7 nucleotides in length. In one embodiment, D and R have a free energy for association from about 5.2 kcal/mole to about 8.2 kcal/mole as measured in the selection buffer conditions, defined below. In another embodiment, D and R have a free energy for association from about 6.0 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions defined below. In yet another embodiment, D and R have a free energy for association from about 7.0 kcal/mole to 8.0 kcal/mole in the selection buffer conditions. In a preferred embodiment, D and R have a free energy for association of 7.5 kcal/mole in the selection buffer conditions described below.

[0072] Q may be a nucleotide sequence from about 10 to about 100 nucleotides in length. In one embodiment, Q is from 10 to about 25 nucleotides in length. In another embodiment, Q is from about 25 to about 50 nucleotides in length. In a further embodiment, Q is from about 50 to about 75 nucleotides in length. In yet another embodiment, Q is from

about 75 to about 100 nucleotides in length. In each embodiment, the nucleotides may be any of the nucleotide bases in DNA or RNA (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA). In one embodiment Q is comprised of DNA bases. In another embodiment, Q is comprised of RNA bases. In yet another embodiment, Q is comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. Alternatively, Q may be a polymer of bifunctional chemical linkers. In one embodiment the bifunctional chemical linker is heterobifunctional. Suitable heterobifunctional chemical linkers include sulfoSMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and lc-SPDP(N-Succinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate). In another embodiment the bifunctional chemical linker is homobifunctional. Suitable homobifunctional linkers include disuccinimidyl suberate, disuccinimidyl glutarate, and disuccinimidyl tartrate. Additional suitable linkers are illustrated in the Examples, such as the phosphoramidate form of Spacer 18 comprised of polyethylene glycol. In one embodiment, Q is from 0 to about 500 angstroms in length. In another embodiment, Q is from about 20 to about 400 angstroms in length. In yet another embodiment, Q is from about 50 to about 250 angstroms in length.

[0073] In a preferred embodiment, A and C are approximately 18 nucleotides in length and have an average GC content of about 60%; B is about 30 to about 60 nucleotides in length; Q is a linker comprising a nucleotide sequence that is from about 10 to 100 nucleotides in length or a bifunctional chemical linker; and D and R range from about 5 to about 7 nucleotides in length and have a free energy of association of about 7.5 kcal/mole.

[0074] As will be appreciated by those of skill in the art, the choice of epitope binding agent, P, can and will vary depending upon the particular target molecule. By way of

example, when the target molecule is a protein P may be an aptamer, or antibody. By way of further example, when P is double stranded nucleic acid the target molecule is typically a macromolecule that binds to DNA or a DNA binding protein. Suitable epitope binding agents, depending upon the target molecule, include agents selected from the group consisting of an aptamer, an antibody, an antibody fragment, a double-stranded DNA sequence, a ligand, a ligand fragment, a receptor, a receptor fragment, a polypeptide, a peptide, a coenzyme, a coregulator, an allosteric molecule, and an ion. In an exemplary embodiment, P is an aptamer sequence ranging in length from about 20 to about 110 bases. In another embodiment, P is an antibody selected from the group consisting of polyclonal antibodies, ascites, Fab fragments, Fab' fragments, monoclonal antibodies, and humanized antibodies. In a preferred embodiment, P is a monoclonal antibody. In an additional embodiment, P is a double stranded DNA.

[0075] Typically in the method, a plurality of nucleic acid constructs, A-B-C-D, are contacted with the epitope bind agent construct, P-Q-R, and the target molecular in the presence of a selection buffer to form a mixture. Several selection buffers are suitable for use in the invention. A suitable selection buffer is typically one that facilitates non-covalent binding of the nucleic acid construct to the target molecule in the presence of the epitope binding agent construct. In one embodiment, the selection buffer is a salt buffer with salt concentrations from about 1mM to 100mM. In another embodiment, the selection buffer is comprised of Tris-HCl, NaCl, KCl, and $MgCl_2$. In a preferred embodiment, the selection buffer is comprised of 50mM Tris-HCl, 100mM NaCl, 5mM KCl, and 1mM $MgCl_2$. In one embodiment, the selection buffer has pH range from about 6.5 to about 8.5. In another embodiment, the selection buffer has a pH range from about 7.0 to 8.0. In a preferred embodiment, the pH is 7.5. Alternatively, the selection buffer may additionally contain

analytes that assist binding of the constructs to the target molecule. Suitable examples of such analytes can include, but are not limited to, protein co-factors, DNA-binding proteins, scaffolding proteins, or divalent ions.

[0076] The mixture of the plurality of nucleic acid constructs, epitope binding agent constructs and target molecules are incubated in selection buffer from about 10 to about 45 min. In yet another embodiment, the incubation is performed for about 15 to about 30 min. Typically, the incubation is performed at a temperature range from about 21° C to about 40° C. In another embodiment, the incubation is performed at a temperature range from about 20°C to about 30°C. In yet another embodiment, the incubation is performed at 35°C. In a preferred embodiment, the incubation is performed at 25°C for about 15 to about 30 min. Generally speaking after incubation, the mixture will typically comprise complexes of the target molecule having nucleic acid construct bound to a first epitope and epitope binding agent construct bound to a second epitope of the target molecule. The mixture will also comprise unbound nucleic acid constructs and epitope binding agent constructs.

[0077] The complex comprising the target molecule having bound nucleic acid construct and bound epitope binding agent construct is preferably isolated from the mixture. In one embodiment, nitrocellulose filters are used to separate the complex from the mixture. In an alternative embodiment magnetic beads are used to separate the complex from the mixture. In yet another embodiment sepharose beads can be used to separate the complex from the mixture. In an exemplary embodiment, strepavidin-linked magnetic beads are used to separate the complex from the mixture.

[0078] Optionally, the target molecules are subjected to denaturation and then the nucleic acid constructs purified from the complex. In one embodiment, urea is used to denature the target molecule. In a preferred embodiment, 7M urea in 1M NaCl is used to

denature the target molecule. The nucleic acid constructs may be purified from the target molecule by precipitation. In another embodiment, the nucleic acid constructs are precipitated with ethanol. In yet another embodiment, the nucleic acid constructs are precipitated with isopropanol. In one embodiment, the precipitated DNA is resuspended in water. Alternatively, the precipitated DNA is resuspended in TE buffer.

[0079] Generally speaking, the purified, resuspended nucleic acid constructs are then amplified using the polymerase chain reaction (PCR). If the nucleic acid construct contains a B comprised of RNA bases, reverse transcriptase is preferably used to convert the RNA bases to DNA bases before initiation of the PCR. The PCR is performed with primers that recognize both the 3' and the 5' end of the nucleic acid constructs in accordance with methods generally known in the art. In one embodiment, either the 3' or 5' primer is attached to a fluorescent probe. In an alternative embodiment, either the 3' or the 5' primer is attached to fluorescein. In another embodiment, either the 3' or 5' primer is biotinylated. In a preferred embodiment, one primer is labeled with fluorescein, and the other primer is biotinylated.

[0080] In addition to primers, the PCR reaction contains buffer, deoxynucleotide triphosphates, polymerase, and template nucleic acid. In one embodiment, the PCR can be performed with a heat-stable polymerase. In a preferred embodiment, the concentrations of PCR reactants are outlined in the examples section as follows: 80uL of dd H2O, 10uL of 10x PCR buffer, 6uL of $MgCl_2$, 0.8uL 25mM dNTPs, 1uL 50uM primer 1(modified with fluorescein), 1uL 50uM primer 2 (biotinylated), 0.5uL Taq polymerase, and 1uL of template.

[0081] In another embodiment, the PCR consists of a warm-up period, where the temperature is held in a range between about 70°C and about 74°C. Subsequently, the PCR consists of several cycles (about 8 to about 25) of 1) incubating the reaction at a temperature

between about 92°C and about 97°C for about 20sec to about 1min; 2) incubating the reaction at a temperature between about 48°C and about 56°C for about 20sec to about 1min; and 3) incubating the reaction at a temperature between about 70°C and about 74°C for about 45sec to about 2min. After the final cycle, the PCR is concluded with an incubation between about 70°C and about 74°C for about 3min to about 10min. In an alternative embodiment, the reaction consists of 12-18 cycles. A preferred embodiment of the PCR, as outlined in the examples section, is as follows: 5 min at 95°C, sixteen cycles of 30s at 95°C, 30s at 50°C, and1min at 72°C, and then an extension period of 5min at 72°C.

[0082] Typically after PCR amplification, the double-stranded DNA PCR product is separated from the remaining PCR reactants. One exemplary embodiment for such separation is subjecting the PCR product to agarose gel electrophoresis. In another embodiment, the PCR product is separated in a low melting point agarose gel. In a preferred embodiment, the gel is a native 10% acrylamide gel made in TBE buffer. In one embodiment, the band(s) having the double-stranded DNA PCR product are visualized in the gel by ethidium bromide staining. In another embodiment, the band(s) are visualized by fluorescein fluorescence. Irrespective of the embodiment, the bands are typically excised from the gel by methods generally known in the art.

[0083] Generally speaking, the double-stranded gel-purified PCR product is separated into single-stranded DNA in accordance with methods generally known in the art. One such embodiment involves using a basic pH to denature the double helix. In another embodiment, 0.15N NaOH is used to denature the helix. In still another embodiment, streptavidin linked beads are used to separate the denatured DNA strands. In a preferred embodiment, magnetic streptavidin beads are used to separate the denatured DNA strands.

[0084] The method of the invention typically involves several rounds of selection, separation, amplification and purification in accordance with the procedures described above until nucleic acid constructs having the desired binding affinity for the target molecule are selected. In accordance with the method, the single-stranded DNA of estimated concentration is used for the next round of selection. In one embodiment, the cycle of selection, separation, amplification, purification, and strand separation is performed from about 4 to about 20 times. In another embodiment, the said cycle is performed from about 12 to about 18 times. In yet another embodiment, the said cycle is performed until the measured binding-activity of the selected nucleic acid constructs reaches the desired strength.

[0085] Alternatively, the single DNA strand attached to the strepavidin-linked beads is used as a template for RNA polymerase. In this embodiment, after the RNA polymerase is finished, the supernatant contains the RNA nucleic acid construct that can be used in another round of RNA aptamer selection.

[0086] In an alternative method, if a RNA aptamer is being selected, the double-stranded, gel-purified PCR DNA product is transcribed with RNA polymerase to produce a single-stranded RNA construct. In such a case, A will typically contain a sequence encoding a promoter recognized by RNA polymerase. In one embodiment, double-stranded, gel-purified PCR DNA product attached to strepavidin-linked beads is used as a template for RNA polymerase. In this embodiment, after the RNA polymerase reaction, the supernatant containing the RNA nucleic acid construct can used in another round of RNA aptamer selection.

[0087] Generally speaking, after the nucleic acid constructs have reached the desired binding specificity, the nucleic acid constructs are cloned, and the cloned DNA is

sequenced. In one embodiment, the sequences are used in aptamer constructs either alone or as part of a molecular biosensor.

(A) Method for Simultaneous Selection of Two or More Aptamers

[0088] Another aspect of the invention is a method for simultaneously selecting two or more aptamers for use in making molecular biosensors having two or more aptamers. The aptamers selected by the method each bind to the same target molecule at two distinct epitopes. Typically, the method comprises contacting a plurality of pairs of nucleic acid constructs with a target molecule to form a mixture. The mixture will generally comprise complexes having target molecule bound with a pair of nucleic acid constructs at distinct epitope sites. According to the method, the complex is isolated from the mixture and the nucleic acid constructs are purified from the complex. The aptamers selected by the method of the invention will comprise the pair of purified nucleic acid constructs.

[0089] In the method of the invention, the first nucleic acid constructs comprises:

i. A-B-C-D

The second nucleic acid construct comprises:

ii. E-F-G-H.

wherein:

A, C, E, and G are each different DNA sequences from about 10 to about 30 nucleotides in length, A and C together comprising a sequence to prime a polymerase chain reaction for amplifying a first aptamer sequence, and E and G together comprising a sequence to prime a polymerase chain reaction for amplifying a second aptamer sequence;

B is a single-stranded nucleotide random sequence from about 20 to about 110 nucleotides in length that contains specific sequences binding to a first epitope of the target molecule;

D and H are a pair of complementary nucleotide sequences from about 2 to about 20 nucleotides in length, wherein D and H have a free energy for association from about 5.5 kcal/mole to about 8.0 kcal/mole at a temperature from approximately 21°C to about 40°C and at a salt concentration of approximately 1 mM to about 100 mM; and

F is a single-stranded nucleotide random sequence from about 20 to about 110 nucleotides in length that contains specific sequences binding to the second epitope of the target molecule.

[0090] In another embodiment, A, C, E and F are each different DNA sequences ranging from about 7 to about 35 nucleotides in length. In another embodiment, A, C, E, and F range from about 15 to about 25 nucleotides in length. In yet another embodiment, A, C, E, and F range from about 15 to about 20 nucleotides in length. In still another embodiment, A, C, E and F range from about 16 to about 18 nucleotides in length. In an exemplary embodiment, A, C, E and F are 18 nucleotides in length. Generally speaking, A, C, E and F have an average GC content from about 53% to 63%. In another embodiment, A, C, E and F have an average GC content from about 55% to about 60%. In a preferred embodiment, A, C, E and F will have an average GC content of about 60%.

[0091] In one embodiment, B and F are single-stranded oligonucleotides synthesized by randomly selecting and inserting a nucleotide base (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA) at every position of the oligonucleotide. In a preferred embodiment, B and F encode an aptamer sequence, such that B binds to the first epitope on the target molecule and F binds to the second epitope on the target molecule. In one embodiment B and F are comprised of DNA bases. In another embodiment, B and F are comprised of RNA bases. In yet another embodiment, B and F are comprised of modified nucleic acid bases, such as modified DNA bases. Examples of suitable modified DNA or

RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. In typical embodiments, B and F are about 20 to 110 nucleotides in length. In another embodiment, B and F are from about 25 to about 75 nucleotides in length. In yet another embodiment, B and F are from about 30 to about 60 nucleotides in length.

[0092] D and H are complementary nucleotide sequences from about 2 to about 20 nucleotides in length. In another embodiment, D and H are from about 4 to about 15 nucleotides in length. In a preferred embodiment, D and H are from about 5 to about 7 nucleotides in length. In one embodiment, D and H have a free energy for association from about 5.2 kcal/mole to about 8.2 kcal/mole as measured in the selection buffer conditions, defined below. In another embodiment, D and H have a free energy for association from about 6.0 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions. In yet another embodiment, D and H have a free energy for association from about 7.0 kcal/mole to 8.0 kcal/mole in the selection buffer conditions. In a preferred embodiment, D and H have a free energy for association of 7.5 kcal/mole in the selection buffer conditions.

[0093] In a preferred embodiment, A, C, E and F are approximately 18 nucleotides in length and have an average GC content of about 60%, B and F are about 30 to about 60 nucleotides in length, and D and H range from about 5 to about 7 nucleotides in length and have a free energy of association of about 7.5 kcal/mole.

[0094] The method for simultaneous selection is initiated by contacting a plurality of pairs of the nucleic acid constructs A-B-C-D and E-F-G-H with the target molecule in the presence of a selection buffer to form a complex. Generally speaking, suitable selection buffers allow non-covalent simultaneous binding of the nucleic acid constructs to the target molecule. The method for simultaneous selection then involves the same steps of selection,

separation, amplification and purification as described in section (a) above involving methods for the selection of an aptamer in the presence of an epitope binding agent construct, with the exception that the PCR is designed to amplify both nucleic acid constructs (A-B-C-D and E-F-G-H), using primers to A, C, E, and F. Typically several rounds of selection are performed until pairs of nucleic acid constructs having the desired affinity for the target molecule are selected. In one embodiment, the cycle of selection, separation, amplification, purification, and strand separation is performed from about 4 to about 20 times. In another embodiment, the cycle is performed from about 12 to about 18 times.

[0095] After the pair of nucleic acid constructs has reached the desired binding specificity, the nucleic acid constructs are cloned, and the cloned DNA is sequenced. The resulting nucleic acid constructs comprise a first aptamer that binds to a first epitope on the target molecule and a second aptamer that binds to a second epitope on the target molecule.

[0096] In another aspect of the invention, two aptamers can be simultaneously selected in the presence of a bridging construct comprised of S-T-U. In one embodiment, S and U are complementary nucleotide sequences from about 2 to about 20 nucleotides in length. In another embodiment, S and U are from about 4 to about 15 nucleotides in length. In a preferred embodiment, S and U are from about 5 to about 7 nucleotides in length. In one embodiment, S and U have a free energy for association from about 5.2 kcal/mole to about 8.2 kcal/mole as measured in the selection buffer conditions, defined below. In another embodiment, S and U have a free energy for association from about 6.0 kcal/mole to about 8.0 kcal/mole as measured in the selection buffer conditions. In yet another embodiment, S and U have a free energy for association from about 7.0 kcal/mole to 8.0 kcal/mole in the selection buffer conditions. In a preferred embodiment, S and U have a free energy for association of 7.5 kcal/mole in the selection buffer conditions.

[0097] T may be a nucleotide sequence from about 10 to about 100 nucleotides in length. In one embodiment, T is from 10 to about 25 nucleotides in length. In another embodiment, T is from about 25 to about 50 nucleotides in length. In a further embodiment, T is from about 50 to about 75 nucleotides in length. In yet another embodiment, T is from about 75 to about 100 nucleotides in length. In each embodiment, the nucleotides may be any of the nucleotide bases in DNA or RNA (A, C, T, G in the case of DNA, or A, C, U, G in the case of RNA). In one embodiment T is comprised of DNA bases. In another embodiment, T is comprised of RNA bases. In yet another embodiment, T is comprised of modified nucleic acid bases, such as modified DNA bases or modified RNA bases. Examples of suitable modified DNA or RNA bases include 2'-fluoro nucleotides, 2'-amino nucleotides, 5'-aminoallyl-2'-fluoro nucleotides and phosphorothioate nucleotides. Alternatively, T may be a polymer of bifunctional chemical linkers. In one embodiment the bifunctional chemical linker is heterobifunctional. Suitable heterobifunctional chemical linkers include sulfoSMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and lc-SPDP(N-Succinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate). In another embodiment the bifunctional chemical linker is homobifunctional. Suitable homobifunctional linkers include disuccinimidyl suberate, disuccinimidyl glutarate, and disuccinimidyl tartrate. Additional suitable linkers are illustrated in the Examples, such as the phosphoramidate form of Spacer 18 comprised of polyethylene glycol. In one embodiment, Q is from 0 to about 500 angstroms in length. In another embodiment, Q is from about 20 to about 400 angstroms in length. In yet another embodiment, Q is from about 50 to about 250 angstroms in length.

[0098] In one embodiment, S is complementary to D and U is complementary to H. In another embodiment, S and U will not bind to D and H unless S, U, D, and H are

brought in close proximity by the A-B-C-D construct and the E-F-G-H construct binding to the target.

[0099] In this embodiment of the invention utilizing the bridging construct, the method is initiated in the presence of nucleic acid constructs A-B-C-D and E-F-G-H, and the bridging construct S-T-U. Generally speaking, the method is performed as described with the same steps detailed above. In one embodiment, after the final round of selection, but before cloning, the bridging construct is ligated to the A-B-C-D construct and the E-F-G-H construct. This embodiment allows the analysis of pairs of selected nucleic acid sequences that are best suited for use in a molecular biosensor.

(c) Selection of Aptamers by *In Vitro* Evolution

[00100] A further aspect of the invention encompasses selection of aptamers by *in vitro* evolution in accordance with methods generally known in the art.

[00101] In another embodiment, the invention is directed to a method of making a set of aptamer constructs, comprising a first and second aptamer construct, comprising the steps of (a) selecting a first aptamer against a first substrate, which comprises a first epitope, and selecting a second aptamer against a second substrate, which comprises a second epitope, wherein the first aptamer is capable of binding to the first epitope and the second aptamer is capable of binding to the second epitope, (b) attaching a first label to the first aptamer and attaching a second label to the second aptamer, (c) attaching a first signaling oligo to the first aptamer and attaching a second signaling oligo to the second aptamer, wherein the second signaling oligo is complementary to the first signaling oligo, and (d) such that (i) the first aptamer construct comprises the first aptamer, the first label and the first signaling oligo, and (ii) the second aptamer construct comprises the second aptamer, the second label and the

second signaling oligo. Preferably, the aptamers are selected using *in vitro* evolution methods, however, natural DNA binding elements may be used in the practice of this invention.

[00102] In a preferred embodiment, the first substrate is a polypeptide and the second substrate is the polypeptide bound to the first aptamer, wherein the first aptamer masks the first epitope, such that the first epitope is not available for the second aptamer to bind. Alternatively, the first aptamer may be replaced by a first aptamer construct that contains (i) the first aptamer and signaling oligo, or (ii) the first aptamer, signaling oligo and label, thereby producing a second substrate that allows for the selection of the optimum second aptamer or aptamer construct for signal detection. As a further step, the first and second aptamer constructs may then be joined together by a flexible linker, as described above.

[00103] In an alternate preferred embodiment, the first substrate is a peptide consisting essentially of the first epitope and the second substrate is a peptide consisting essentially of the second epitope. Thus, in this alternate embodiment, there is no need to mask an epitope in the production or selection of aptamers. Again, the first and second aptamer constructs created by this method may be linked together by a flexible linker, as described above.

III. Uses of the Molecular Biosensors

[00104] A further aspect of the invention encompasses the use of the molecular biosensors of the invention in several applications. In certain embodiments, the molecular biosensors are utilized in methods for detecting one or more target molecules. In other

embodiments, the molecular biosensors may be utilized in kits and for therapeutic applications.

[00105] In one embodiment, the molecular biosensors may be utilized for the detection of a target molecule. The method generally involves contacting a molecular biosensor of the invention with the target molecule. To detect a target molecule, the method typically involves target-molecule induced co-association of two epitope-binding agents (present on the molecular biosensor of the invention) that each recognize distinct epitopes on the target molecule. The epitope-binding agents each comprise complementary signaling oligonucleotides that are labeled with detection means and are attached to the epitope binding agents through a flexible linker. Co-association of the two epitope-binding agents with the target molecule results in bringing the two signaling oligonucleotides into proximity such that a detectable signal is produced. Typically, the detectable signal is produced by any of the detection means known in the art or as described herein.

[00106] In one particular embodiment, a method for the detection of a target molecule that is a protein or polypeptide is provided. The method generally involves detecting a polypeptide in a sample comprising the steps of contacting a sample with a molecular biosensor of the invention. By way of non limiting example, several useful bivalent molecular biosensors are illustrated in **FIG. 24**. Panel 24A depicts a molecular biosensor comprising two aptamers recognizing two distinct epitopes of a protein. Panel 24B depicts a molecular biosensor comprising a double stranded polynucleotide containing binding site for DNA binding protein and an aptamer recognizing a distinct epitope of the protein. Panel 24C depicts a molecular biosensor comprising an antibody and an aptamer recognizing distinct epitopes of the protein. Panel 24D depicts a molecular biosensor comprising a double stranded polynucleotide containing a binding site for a DNA binding

protein and an antibody recognizing a distinct epitope of the protein. Panel 24E depicts a molecular biosensor comprising two antibodies recognizing two distinct epitopes of the protein. Panel 24F depicts a molecular biosensor comprising two double stranded polynucleotide fragments recognizing two distinct sites of the protein. Panel 24G depicts a molecular biosensor comprising two single stranded polynucleotide elements recognizing two distinct sequence elements of another single stranded polynucleotide. Panel 24H depicts a molecular biosensor that allows for the direct detection of formation of a protein-polynucleotide complex using a double stranded polynucleotide fragment (containing the binding site of the protein) labeled with a first signaling oligonucleotide and the protein labeled with a second signaling oligonucleotide. Panel 24I depicts a molecular biosensor that allows for the direct detection of the formation of a protein-protein complex using two corresponding proteins labeled with signaling oligonucleotides.

[00107] Optionally, the invention also encompasses a solid surface having the molecular constructs of the invention attached thereto. For example, the first aptamer construct may be fixed to a surface, the second aptamer construct may be fixed to a surface, or both may be fixed to a surface. Non-limiting examples of suitable surfaces include microtitre plates, test tubes, beads, resins and other polymers. In a preferred embodiment, the first aptamer construct and the second aptamer construct may be joined with each other by a flexible linker to form a bivalent aptamer. Preferred flexible linkers include Spacer 18 polymers and deoxythymidine ("dT") polymers.

[00108] In another embodiment, the molecular biosensors may be used to detect a target molecule that is a macromolecular complexes in a sample. In this embodiment, the first epitope is preferably on one polypeptide and the second epitope is on another polypeptide, such that when a macromolecular complex is formed, the one and another

polypeptides are bought into proximity, resulting in the stable interaction of the first aptamer construct and the second aptamer construct to produce a detectable signal, as described above. Also, the first and second aptamer constructs may be fixed to a surface or to each other via a flexible linker, as described above.

[00109] In another embodiment, the molecular biosensors may be used to detect a target molecule that is an analytes in a sample. In this embodiment, when the analyte is bound to a polypeptide or macromolecular complex, a first or second epitope is created or made available to bind to a first or second aptamer construct. Thus, when an analyte is present in a sample that contains its cognate polypeptide or macromolecular binding partner, the first aptamer construct and the second aptamer construct are brought into stable proximity to produce a detectable signal, as described above. Also, the first and second aptamer constructs may be fixed to a surface or to each other via a flexible linker, as described above.

[00110] Alternatively, in certain embodiments it is contemplated that the molecular biosensor may not include a detections means. By way of example, when the molecular biosensor is a bivalent aptamer construct, the bivalent aptamer construct may not have labels for detection. It is envisioned that these alternative bivalent aptamer constructs may be used much like antibodies to detect molecules, bind molecules, purify molecules (as in a column or pull-down type of procedure), block molecular interactions, facilitate or stabilize molecular interactions, or confer passive immunity to an organism. It is further envisioned that the bivalent aptamer construct can be used for therapeutic purposes. This invention enables the skilled artisan to build several combinations of aptamers that recognize any two or more disparate epitopes form any number of molecules into a bivalent, trivalent, or other multivalent aptamer construct to pull together those disparate molecules to test the effect or to produce a desired therapeutic outcome. For example, a bivalent aptamer construct may be

constructed to facilitate the binding of a ligand to its receptor in a situation wherein the natural binding kinetics of that ligand to the receptor is not favorable (e.g., insulin to insulin receptor in patients suffering diabetes.)

[00111] In another embodiment, the invention is directed to a kit comprising a first epitope binding agent, to which is attached a first label, and a second epitope binding agent, to which is attached a second label, wherein (a) when the first epitope binding agent and the second epitope binding agent label bind to a first epitope of a polypeptide and a second epitope of the polypeptide, respectively, (b) the first label and the second label interact to produce a detectable signal. In a preferred embodiment the epitope binding agent is an aptamer construct, which comprises an aptamer, a label and a signaling oligo. However, the epitope binding agent may be an antibody or antibody fragment. The kit is useful in the detection of polypeptides, analytes or macromolecular complexes, and as such, may be used in research or medical/veterinary diagnostics applications.

[00112] In yet another embodiment, the invention is directed to a method of diagnosing a disease comprising the steps of (a) obtaining a sample from a patient, (b) contacting the sample with a first epitope binding agent and a second epitope binding agent, and (c) detecting the presence of a polypeptide, analyte or macromolecular complex in the sample using a detection method, wherein the presence of the polypeptide, analyte or macromolecular complex in the sample indicates whether a disease is present in the patient. In a preferred embodiment, (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide

and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs. Preferred samples include blood, urine, ascites, cells and tissue samples/biopsies. Preferred patients include humans, farm animals and companion animals.

[00113] In yet another embodiment, the invention is directed to a method of screening a sample for useful reagents comprising the steps of (a) contacting a sample with a first epitope binding agent and a second epitope binding agent, and (b) detecting the presence of a useful reagent in the sample using a detection method. Preferred reagents include a polypeptide, which comprises a first epitope and a second epitope, an analyte that binds to a polypeptide (in which case the method further comprises the step of adding the polypeptide to the screening mixture) and a potential therapeutic composition. In a preferred embodiment, (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs.

DEFINITIONS

[00114] As used herein, the term "analyte" refers generally to a ligand, chemical moiety, compound, ion, salt, metal, enzyme, secondary messenger of a cellular signal

transduction pathway, drug, nanoparticle, environmental contaminant, toxin, fatty acid, steroid, hormone, carbohydrate, amino acid, peptide, polypeptide, protein or other amino acid polymer, microbe, virus or any other agent which is capable of binding to a polypeptide, protein or macromolecular complex in such a way as to create an epitope or alter the availability of an epitope for binding to an aptamer.

[00115] The term "antibody" generally means a polypeptide or protein that recognizes and can bind to an epitope of an antigen. An antibody, as used herein, may be a complete antibody as understood in the art, i.e., consisting of two heavy chains and two light chains, or be selected from a group comprising polyclonal antibodies, ascites, Fab fragments, Fab' fragments, monoclonal antibodies, humanized antibodies, and a peptide comprising a hypervariable region of an antibody.

[00116] The term "aptamer" refers to a polynucleotide, generally a RNA or a DNA that has a useful biological activity in terms of biochemical activity, molecular recognition or binding attributes. Usually, an aptamer has a molecular activity such as binding to a target molecule at a specific epitope (region). It is generally accepted that an aptamer, which is specific in its binding to any polypeptide, may be synthesized and/or identified by in vitro evolution methods.

[00117] As used herein, "detection method" means any of several methods known in the art to detect a molecular interaction event. The phrase "detectable signal", as used herein, is essentially equivalent to "detection method." Detection methods include detecting changes in mass (e.g., plasmin resonance), changes in fluorescence (e.g., FRET, FCCS, fluorescence quenching or increasing fluorescence, fluorescence polarization, flow cytometry), enzymatic activity (e.g., depletion of substrate or formation of a product, such as a detectable dye – NBT-BCIP system of alkaline phosphatase is an example), changes in

chemiluminescence or scintillation (e.g., scintillation proximity assay, luminescence resonance energy transfer, bioluminescence resonance energy transfer and the like), and ground-state complex formation, excimer formation, colorimetric substance detection, phosphorescence, electro-chemical changes, and redox potential changes.

[00118] The term "epitope" refers generally to a particular region of a target molecule. Examples include an antigen, a hapten, a molecule, a polymer, a prion, a microbe, a cell, a peptide, polypeptide, protein, or macromolecular complex. An epitope may consist of a small peptide derived from a larger polypeptide. An epitope may be a two or three-dimensional surface or surface feature of a polypeptide, protein or macromolecular complex that comprises several non-contiguous peptide stretches or amino acid groups.

[00119] The term "epitope binding agent" refers to a substance that is capable of binding to a specific epitope of an antigen, a polypeptide, a protein or a macromolecular complex. Non-limiting examples of epitope binding agents include aptamers, double-stranded DNA sequence, ligands and fragments of ligands, receptors and fragments of receptors, antibodies and fragments of antibodies, polynucleotides, coenzymes, coregulators, allosteric molecules, and ions.

[00120] The term "epitope binding agent construct" refers to a construct that contains an epitope-binding agent and can serve in a "molecular biosensor" with another molecular biosensor. Preferably, an epitope binding agent construct also contains a "linker," and a "signaling oligo". Epitope binding agent constructs can be used to initiate the aptamer selection methods of the invention. A first epitope binding agent construct and a second epitope binding agent construct may be joined together by a "linker" to form a "bivalent epitope binding agent construct." An epitope binding agent construct can also be referred to

as a molecular recognition construct. An aptamer construct is a special kind of epitope binding agent construct wherein the epitope binding agent is an aptamer.

[00121] The phrase "*in vitro* evolution" generally means any method of selecting for an aptamer that binds to a biomolecule, particularly a peptide or polypeptide. *In vitro* evolution is also known as "*in vitro* selection", "SELEX" or "systematic evolution of ligands by exponential enrichment." Briefly, *in vitro* evolution involves screening a pool of random polynucleotides for a particular polynucleotide that binds to a biomolecule or has a particular activity that is selectable. Generally, the particular polynucleotide (i.e., aptamer) represents a very small fraction of the pool, therefore, a round of aptamer amplification, usually via polymerase chain reaction, is employed to increase the representation of potentially useful aptamers. Successive rounds of selection and amplification are employed to exponentially increase the abundance of the particular and useful aptamer. *In vitro* evolution is described in Famulok, M.; Szostak, J. W., *In Vitro* Selection of Specific Ligand Binding Nucleic Acids, *Angew. Chem.* 1992, *104*, 1001. (*Angew. Chem. Int. Ed. Engl.* 1992, *31*, 979-988.); Famulok, M.; Szostak, J. W., Selection of Functional RNA and DNA Molecules from Randomized Sequences, *Nucleic Acids and Molecular Biology*, Vol 7, F. Eckstein, D. M. J. Lilley, Eds., Springer Verlag, Berlin, 1993, pp. 271; Klug, S.; Famulok, M., All you wanted to know about SELEX; *Mol. Biol. Reports* 1994, *20*, 97-107; and Burgstaller, P.; Famulok, M. Synthetic ribozymes and the first deoxyribozyme; *Angew. Chem.* 1995, *107*, 1303-1306 (*Angew. Chem. Int. Ed. Engl.* 1995, *34*, 1189-1192), which are incorporated herein by reference.

[00122] In the practice of certain embodiments of the invention, *in vitro* evolution is used to generate aptamers that bind to distinct epitopes of any given polypeptide or macromolecular complex. Aptamers are selected against "substrates", which contain the

epitope of interest. As used herein, a "substrate" is any molecular entity that contains an epitope to which an aptamer can bind and that is useful in the selection of an aptamer.

[00123] The term "label", as used herein, refers to any substance attachable to a polynucleotide, polypeptide, aptamer, nucleic acid component, or other substrate material, in which the substance is detectable by a detection method. Non-limiting examples of labels applicable to this invention include but are not limited to luminescent molecules, chemiluminescent molecules, fluorochromes, fluorescent quenching agents, colored molecules, radioisotopes, scintillants, massive labels (for detection via mass changes), biotin, avidin, streptavidin, protein A, protein G, antibodies or fragments thereof, Grb2, polyhistidine, Ni^{2+}, Flag tags, myc tags, heavy metals, enzymes, alkaline phosphatase, peroxidase, luciferase, electron donors/acceptors, acridinium esters, and colorimetric substrates. The skilled artisan would readily recognize other useful labels that are not mentioned above, which may be employed in the operation of the present invention.

[00124] As used herein, the term "macromolecular complex" refers to a composition of matter comprising a macromolecule. Preferably, these are complexes of one or more macromolecules, such as polypeptides, lipids, carbohydrates, nucleic acids, natural or artificial polymers and the like, in association with each other. The association may involve covalent or non-covalent interactions between components of the macromolecular complex. Macromolecular complexes may be relatively simple, such as a ligand bound polypeptide, relatively complex, such as a lipid raft, or very complex, such as a cell surface, virus, bacteria, spore and the like. Macromolecular complexes may be biological or non-biological in nature.

[00125] The term "molecular biosensor" and "molecular beacon" are used interchangeably herein to refer to a construct comprised of at least two epitope binding agent

constructs. The molecular biosensor can be used for detecting or quantifying the presence of a target molecule using a chemical-based system for detecting or quantifying the presence of an analyte, a prion, a protein, a nucleic acid, a lipid, a carbohydrate, a biomolecule, a macromolecular complex, a fungus, a microbial organism, or a macromolecular complex comprised of biomolecules using a measurable read-out system as the detection method.

[00126] The phrase "natural cognate binding element sequence" refers to a nucleotide sequence that serves as a binding site for a nucleic acid binding factor. Preferably the natural cognate binding element sequence is a naturally occurring sequence that is recognized by a naturally occurring nucleotide binding factor.

[00127] The term "nucleic acid construct" refers to a molecule comprising a random nucleic acid sequence flanked by two primers. Preferably, a nucleic acid construct also contains a signaling oligo. Nucleic acid constructs are used to initiate the aptamer selection methods of the invention.

[00128] The term "signaling oligo" means a short (generally 2 to 15 nucleotides, preferably 5 to 7 nucleotides in length) single-stranded polynucleotide. Signaling oligos are typically used in pairs comprising a first signaling oligo and a second signaling oligo. Preferably, the first signaling oligo sequence is complementary to the second signaling oligo. Preferably, the first signaling oligo and the second signaling oligo can not form a stable association with each other through hydrogen bonding unless the first and second signaling oligos are brought into close proximity to each other through the mediation of a third party agent.

EXAMPLES

The following examples illustrate the invention.

example 1: General method for preparing specific aptamer constructs

Introduction

[00129] Disclosed is a method for the rapid and sensitive detection in a sample of proteins, protein complexes, or analytes that bind to proteins. This method is based on the protein-driven association of two constructs containing aptamers that recognize two distinct epitopes of a protein (a.k.a. "aptamer constructs") (Fig. 1A). These two aptamer constructs contain short complementary signaling oliginucleotides attached to the aptamers through a flexible linker. Upon the simultaneous binding of the two aptamers to the target protein, the complementary oligonucleotides (a.k.a. "signaling oligos") are brought into relative proximity that promotes their association to form a stable duplex. Attaching fluorescence probes to the ends of the signaling oligos provides a means of detecting the protein-induced association of the two aptamer constructs (Fig. 1A). In the case of proteins that possess natural nucleic acid binding activity, one of the aptamers can be substituted with a nucleic acid sequence containing the DNA-binding sequence that the protein naturally binds to protein (Fig. 1B).

[00130] Development or selection of aptamers directed to two distinct epitopes of a given protein is an essential step in developing the aptamer constructs depicted in Fig. 1. Review of the available literature on aptamers reveals at least two possible approaches to achieve this goal. The first approach is to perform *in vitro* selection (a.k.a. *in vitro* evolution) of nucleic acid aptamers using different methods for the separation of protein-bound and protein-unbound nucleic acid aptamers. The rationale here is that in these different partitioning methods different regions of the protein are preferentially displayed resulting in aptamers directed to different regions of the protein surface. Aptamers selected to thrombin (infra) are an example of such an approach.

[00131] The *in vitro* selection of a first aptamer using as a substrate thrombin immobilized on agarose beads resulted in an aptamer binding the thrombin at the heparin exosite. Additional in vitro selection using as a substrate the thrombin-first aptamer complex, which was bound to nitrocellulose as the partitioning method, resulted in a second aptamer binding the thrombin at the fibrinogen exosite.

[00132] While useful, this partitioning approach relies on the chance selection of distinct epitopes rather than on intelligent design. The second approach is to raise or select the aptamers using as substrates peptides that correspond to selected regions of the target protein molecule. There is evidence in the art, which demonstrates that such strategy can be used to develop aptamers capable of recognizing the intact protein from which the peptide used as a substrate for aptamer development was derived. Furthermore, this approach has been widely used to generate antibodies, which recognize an intact protein.

[00133] The general approach for preparing a set of aptamers directed to an epitope of the protein distinct from the binding site of the first aptamer can be also used for proteins that possess natural DNA binding activity. That is, co-aptamers, which bind the substrate protein at a site distinct from the natural DNA binding site, can be produced. Co-aptamers produced by this method are optimized for functioning in the molecular detection method depicted in Fig.1.

Results and Discussion

[00134] Figure 2 summarizes five possible methods for selecting aptamers useful in the practice of the invention. Panel A depicts the selection of an additional aptamer in the presence of a target bound to a known aptamer. The nucleic acid construct is comprised of a signaling oligo, represented by the blue bar, and two primers flanking a random DNA

sequence. In practice, the signaling oligo is treated as a specific subpart of the primer in the nucleic acid construct. A complimentary signaling oligo is attached to the pre-selected aptamer via a long flexible linker. Here, the process begins by combining the nucleic acid construct, the target, and the known aptamer construct. Selection of aptamers using such a random DNA (or RNA) construct will be biased towards aptamers capable of binding to the target at an epitope distinct from the epitope of the known aptamer construct, and that will function in molecular biosensors depicted in Fig. 3B.

[00135] An alternative scenario is depicted in panel B, which describes the simultaneous selection of two aptamers binding two distinct epitopes of the target. The nucleic acid constructs are comprised of signaling oligos (represented by the blue bars at the end of primer 1 and primer 4) and two primers flanking either side of a random-sequence. There are at least two different types of nucleic acid constructs, each type containing unique primer sequences. In panel B, one type contains primers 1and 2, and the second contains primers 3 and 4. In this example, the process begins with combining both types of nucleic acid constructs, and the target. Selection of aptamers using such random DNA (or RNA) constructs will be biased towards aptamers capable of binding to the target simultaneously at two distinct epitopes of the protein, and that will function in sensors depicted in Fig. 3B.

[00136] Panel C depicts an alternative design for simultaneous selection of two aptamers binding two distinct epitopes of the target. In addition to the two different types of nucleic acid constructs, a third bridging construct is used. The bridging construct comprises an additional pair of short oligonucleotides (blue bars) connected by a flexible linker. These oligonucleotides will be complementary to the short oligonucleotides at the end of the nucleic acid constructs. The presence of the bridging construct during selection will provide a bias towards selecting pairs of aptamers capable of simultaneously binding the target. Before

cloning of the selected aptamers (after the last selection) the pairs of selected sequences will be enzymatically ligated using T4 ligase to preserve the information regarding the preferred pairs between various selected aptamers.

[00137] In a fourth alternate embodiment, a second aptamer can be selected in the presence of a target bound by an antibody (Figure 2D). The signaling oligo in the nucleic acid construct, depicted by the blue bar, is complementary to the signaling oligo attached to the antibody via a long flexible linker. The process begins by combining the nucleic acid construct, the target, and the antibody construct. Selection of an aptamer using such a random DNA (or RNA) construct will be biased towards aptamers able to bind to the protein at an epitope distinct from the antibody epitope and will function in sensors depicted in Fig. 24C.

[00138] In a fifth alternate embodiment, a second aptamer can be selected in the presence of the target bound to a double-stranded DNA fragment (Figure 2E). The signaling oligo in the nucleic acid construct, depicted by the blue bar, is complementary to the signaling oligo attached to the double-stranded DNA construct via a long flexible linker. The process begins by combining the nucleic acid construct, the target, and the double-stranded DNA construct. Selection of an aptamer using such a random DNA (or RNA) construct will be biased towards aptamers able to bind to the target at a site distinct from the double-stranded DNA binding site and will function in sensors depicted in Fig. 1B or 24B.

example 2: Methods and aptamers for detecting thrombin

Introduction

[00139] The inventors of the instant invention have developed a methodology for detecting DNA binding proteins, as described in Heyduk, T. and Heyduk, E. "Molecular beacons for detecting DNA binding proteins," *Nature Biotechnology*, **20,** 171-176, 2002, Heyduk, E., Knoll, E., and Heyduk, T. "Molecular beacons for detecting DNA binding

proteins: mechanism of action," *Analyt. Biochem.* **316,** 1-10, 2003, and copending patent applications number 09/928,385, which issued as U.S. Pat. No. 6,544,746, 10/062,064, PCT/US02/24822 and PCT/US03/02157, all of which are incorporated herein by reference. This methodology is based on splitting the DNA binding site for a protein into two DNA "half-sites" (Fig. 3A). Each of the resulting "half-sites" contains a short complementary single-stranded region of the length designed to introduce some propensity for the two DNA "half-sites" to associate recreating the duplex containing the fully functional cognate protein binding site. This propensity is designed to be low such that in the absence of the protein only a small fraction of DNA half-sites will associate. When the protein is present in the reaction mixture, it will bind only to the duplex containing a full and functional binding site. This selective binding drives the association of DNA half-sites and this protein-dependent association can be used to generate a spectroscopic or other signal reporting the presence of the target protein.

[00140] The term "molecular beacons" is used in the scientific literature to describe this assay in order to emphasize the fact that the selective recognition and generation of the reporting signal occur simultaneously. Molecular beacons for DNA binding proteins have been developed for several proteins (Heyduk and Heyduk, 2002) illustrating their general applicability. Their physical mechanism of action has been established (Heyduk, Knoll and Heyduk, 2003) and they have also been used as a platform for the assay detecting the presence of ligands binding to DNA binding proteins (Heyduk, E., Fei, Y., and Heyduk, T. Homogenous fluorescence assay for cAMP. *Combinatorial Chemistry and High-throughput Screening* **6**, 183-194, 2003). While already very useful, this assay is limited to proteins, which exhibit natural DNA binding activity.

[00141] It has been well established that nucleic acid (DNA or RNA) aptamers capable of specific binding to proteins lacking natural DNA binding activity can be produced by in vitro selection methods (Ellington, A.D., and Szostak, J.W. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822, 1990; Tuerk, C., and Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505-510, 1990; Gold, L., Polisky, B., Uhlenbeck, O. & Yarus, M. Diversity of Oligonucleotide Function. *Ann. Rev. Biochem.* **64**, 763-797, 1995; and Wilson, D.S. & Szostak, J.W. In vitro selection of functional nucleic acids. *Ann. Rev. Biochem.* **68**, 611-647, 1999; all of which are incorporated herein by reference). In vitro selection involves selection of nucleic acid sequences, which bind to a specific substrate target, from a pool of random DNA sequences by cycles of binding, washing out unbound sequences and PCR amplification of target-bound sequences. Numerous examples of the successful selection of aptamers that specifically bind to a variety of proteins as well as other target molecules (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Gold et alia, 1995; Wilson and Szostak, 1999) provide a strong indication that producing aptamers to any and all proteins is possible.

[00142] Described in this example is the novel concept of nucleic acid-based molecular beacons for protein detection, which is not limited to proteins with natural DNA binding activity. The example of thrombin (infra) provides experimental validation for this invention.

Results and Discussion

[00143] Fig. 3 illustrates the overall concept of molecular beacons recognizing any target protein. This design shares some general similarities with molecular beacons for DNA binding proteins described previously and supra (Fig. 3A). Instead of splitting the DNA

duplex containing the natural binding site for a protein into the two "half-sites", two aptamers recognizing two different epitopes of the protein are used as functional equivalents of the "half-sites". Short complementary oligonucleotides (signaling oligos) containing the fluorophore (first label) and the quencher (second label) are attached to the two aptamers via a flexible linker (Fig. 3B). In the absence of the target protein, the two aptamer constructs do not associate since the complementary signal oligos are too short to promote association. In the presence of the target protein, the preferential binding of the protein to the two aptamers should drive the association of the two aptamer constructs resulting in a fluorescence signal change due to bringing the first and second labels into a close proximity.

[00144] Thrombin was selected as a model non-DNA-binding-protein system to provide experimental verification of the concept illustrated in Fig. 3B. Two laboratories have previously identified DNA aptamers that selectively recognized two distinct epitopes of the protein (Bock, L.C., Griffin, L.C., Latham, J.A., Vermass, E.H., and Toole, J.J. Selection of single-stranded DNA molecules that bind and inhibit human thrombin, *Nature* **355,** 564-566, 1992; and Tasset, D.M., Kubik, M.F., and Steiner, W. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes, *J. Mol. Biol.* **272,** 688-98, 1997, which are incorporated herein by reference). Oligonucleotide constructs used herein are listed in Table 1. One aptamer (G15D; THR4 in Fig. 4) was shown to bind to the heparin exosite whereas the other aptamer (60-18 [29]; THR3 in Fig. 4) was shown to bind to the fibrinogen exosite. As a first step towards developing a set of aptamer constructs useful for recognizing thrombin, we prepared various aptamer constructs in which the above aptamers were covalently linked by flexible linkers (Fig. 4). The primary purpose of these experiments was to determine if indeed linking the two aptamer constructs with a flexible linker would produce a bivalent aptamer construct capable of binding thrombin with higher affinity compared to a set of individual

aptamer constructs. A second purpose of these experiments was to establish a suitable length of the linker and the appropriate orientation of 5' and 3' ends of the two aptamers with respect to the linker.

[00145] Individual aptamers were labeled with fluorescein (THR1 and THR2 in Fig. 4) to facilitate determination of the affinity of various constructs for thrombin. Formation of a complex between thrombin and fluorescein-labeled 60-18 [29] aptamer (THR1) could be conveniently followed by fluorescence polarization (Fig. 5A) whereas binding of the fluorescein-labeled G15D aptamer (THR2) could be followed by changes in fluorescence intensity (Fig. 5B). Both aptamers bound thrombin in the nanomolar concentration range (Fig. 5A and 5B). Quantitative analysis of the binding in the case of THR2 (Fig. 5C) returned the value of K_d of 6.3 nM. This is somewhat of a higher affinity than that previously suggested (Bock et alia, 1992), which could be explained by the true equilibrium binding assay used by us vs. the non-equilibrium methodology used previously. When the binding of THR2 was performed in the presence of 10-fold excess of unlabeled 60-18 [29] aptamer (THR3) (Fig. 5D) only a small and insignificant decrease in affinity was observed. This shows that indeed G15D and 60-18 [29] aptamers bind independently to two distinct epitopes of thrombin.

[00146] In the next step the ability of various aptamer constructs illustrated in Fig. 4 to compete with THR2 for binding to thrombin was evaluated. Fig. 6 illustrates the manner in which these experiments were performed. Fluorescence spectra of HR2 were recorded in the presence and absence of thrombin (Fig. 6A). Thrombin produced ~50% increase in fluorescence of THR2. Unlabeled competitor aptamer constructs were then added (Figs. 6 B-D). A small effect of thrombin on the fluorescence of THR2 in the presence of a competitor would be a hallmark of an efficient competitor. THR3 was not a competitor (Fig. 6B) in

agreement with the data shown in Fig. 5 C and D. THR4 (an unlabeled variant of THR2) was able to compete as expected (Fig. 6C). However, THR7 (one of the bivalent aptamer constructs) was a much better competitor than THR4 (Fig. 6D). No fluorescence change of THR2 in the presence of thrombin was detected when THR7 was present in solution. Fig. 7 shows a summary of the competition experiments with all of the constructs shown in Fig. 4.

[00147] All bivalent aptamer constructs were shown to bind to thrombin much tighter (K_d's in pM range) than individual aptamers, thus providing validation of the expectation that linking two aptamers, which recognize two different epitopes of the protein, with flexible linkers should produce high-affinity thrombin ligands. Additionally, these data showed that linking two aptamers by a longer linker containing 10 Spacer18 units produced slightly better affinity for thrombin (compare binding of THR5 vs. THR6). Also, these data showed that orientation of the aptamers with respect to the linker as in THR7 produced better affinity (compare affinity of THR6 vs. THR7). Thus, in all subsequent experiments constructs having an aptamer orientation as in THR7 were used.

[00148] The purpose of the experiments shown in Fig. 8 was to demonstrate that both epitopes of thrombin are important for high affinity binding of bivalent aptamer constructs. Direct competition between binding of THR2 and the bivalent aptamer construct provided evidence that the epitope recognized by THR2 (heparin exosite) was necessary for bivalent aptamer binding. To demonstrate that the second epitope was also important, we compared the ability of a bivalent aptamer construct (THR5, see Fig. 4) to compete with THR2 for binding to thrombin in the absence and presence of excess of unlabeled THR3. We expected that if THR5 needs both thrombin epitopes for high-affinity binding, in the presence of THR3 one should observe diminished ability of THR5 to compete with THR2. This is exactly what has been observed in experiments illustrated in Fig. 8. THR5 alone was a very

effective competitor for THR2 (compare Fig. 8D with 8A). THR3 alone was not a competitor for THR2 (compare Fig. 8A and C). THR5 in the presence of THR3 was a worse competitor than THR5 alone (compare Fig. 8B with 8C). We therefore concluded that high-affinity binding of the bivalent aptamer constructs to thrombin involves both first and second aptamer epitopes.

[00149] The bivalent aptamer construct-thrombin complex was stable enough to survive electrophoresis in native polyacrylamide gel (Fig. 9A). We took advantage of this attribute to determine the stoichiometry of the complex using EMSA to follow THR7-thrombin complex formation. We performed a titration of THR7 with thrombin at high concentrations of both molecules. Under these conditions, the binding should be stoichiometric. The plot of the complex formed vs. the ratio of thrombin to THR7 did in fact show a 1:1 stoichiometry of the complex (Fig. 9B).

[00150] The experiments illustrated in Fig. 10 and 11 were performed to test if an alternative design of bivalent aptamer constructs could be used to prepare these constructs. We designed bivalent aptamer constructs shown in Fig. 10 such that they were made entirely of DNA, avoiding the use of non-DNA linker (poly dT was used as the linker in this case) (Fig. 10). This could potentially offer more flexibility in designing such constructs and could also lower the cost of making the aptamer constructs. Two aptamers were joined together by a DNA duplex at the end of flexible linkers (Fig. 10). This aspect of the invention was intended to mimic the design of signaling "beacons" (Fig. 3B) in which the signaling function involves formation of a DNA duplex at the end of the linkers connecting the aptamers to the duplex. Three different lengths of the poly dT linker were tested (7, 17 and 27 nt) to determine the minimal linker length requirement for high-affinity binding. Fig. 11 shows the results of simultaneous titration of the constructs shown in Fig. 10 with thrombin. Formation

of aptamer construct-thrombin complexes was followed by EMSA. Each of the constructs bound thrombin with high affinity. However, it is clear that the construct with 7 nt poly dT linker had significantly lower affinity to thrombin compared to constructs with 17 and 27 nt linkers. This is best illustrated by inspecting the lane marked with the asterisk which shows that at this particular concentration of thrombin almost all of 17 and 27 nt poly dT linker constructs were bound by thrombin whereas a significant (~50%) fraction of the 7 nt poly dT construct remained unbound. In summary, the results described in Figs. 10 and 11 show that the alternative design of bivalent aptamer constructs illustrated in Fig. 10 is feasible and that at least 17 nt long poly dT linker connecting the aptamers with the DNA duplex is more optimal for binding of the constructs to thrombin.

[00151] The experimental data presented in Figs. 3-11 provided evidence that all necessary conditions for the signaling beacon shown in Fig. 3B to function were met in the case of thrombin and the two aptamers binding to two distinct region of thrombin. Based on the information provided by the experiments illustrated in Figs. 3-11, we designed and tested a thrombin signaling beacon. The beacon shown in Fig. 12A and B is a derivative of THR16/THR17 bivalent aptamer construct. Aptamers were connected using 17 nt long poly dT linker to 7 nt complementary oligonucleotides (signaling oligos) labeled at 5' and 3' with fluorescein and dabcyl, respectively. Addition of thrombin to a mixture of THR8 and THR9 resulted in protein-dependent quenching of fluorescence intensity (Fig. 12C). No fluorescence change was observed upon addition of thrombin to THR9 in the absence of dabcyl-labeled partner (THR8) (Fig. 12C). Clearly, these data show that indeed the expected thrombin-driven association between THR8 and THR9 (as illustrated in Fig. 12B) was observed and a functional thrombin signaling beacon was thus obtained.

[00152] The magnitude of the fluorescence change induced by thrombin, while very reproducible and specific, initially was not very large (~ 20%). We therefore sought to improve this property of the thrombin signaling beacon by replacing the poly dT linkers with the more flexible Spacer 18 linker (Fig. 13A&B). We reasoned that poly dT linkers, while flexible, exhibit some residual rigidity (Mills, J.B., Vacano, E., and Hagerman, P.J. Flexibility of single-stranded DNA: use of gapped duplex helices to determine the persistence lengths of poly(dT) and poly(dA), *J. Mol. Biol.* **285**, 245-57, 1999; which is incorporated herein by reference), which could impede the association of the signaling duplex when the two aptamers are bound to thrombin. The beacon shown in Fig. 13 differs only in the nature of the linkers from the beacon shown in Fig. 12. The remaining sequence is otherwise identical. Fig. 13 C shows that upon addition of thrombin to a mixture of THR20 and THR21, protein concentration-dependent quenching of fluorescence was observed whereas no change of fluorescence was detected when thrombin was added to THR21 alone. Response of the beacon to thrombin in the case of this particular beacon was much larger (a ~ 2-fold decrease in fluorescence). The degree of fluorescence signal change in this case was comparable to what we had previously observed with beacons for detecting DNA binding proteins (supra). We concluded thus that a functional thrombin beacon was obtained and that the design utilizing a more flexible Spacer18 linker resulted in a better signal change upon thrombin binding compared to the design with poly dT linker. We next conducted a series of experiments to further characterize the behavior of this thrombin beacon.

[00153] The experiment illustrated in Fig. 14 was conducted to provide confirmation that indeed the fluorescein-labeled aptamer construct (THR21) was incorporated into a stable complex in the presence of THR20 and thrombin. Fig. 15 shows the results, which illustrates the sensitivity of thrombin detection (Fig. 15A) and specificity of

thrombin detection (Fig. 15B). Because the binding of thrombin to bivalent aptamer constructs was extremely tight (pM K_d's), and since the assay appears to be limited only by the sensitivity of detection of fluorescein signal, the sensitivity of thrombin detection could be manipulated by changing the concentration of the aptamer constructs. This is illustrated in Fig. 15 A where using 50 nM THR21 and 75 nM THR20, ~ 10 nM of thrombin could be detected whereas, when 10 fold smaller concentrations of aptamer constructs were used (5 nM THR21 and 7.5 nM THR20), a 10 fold lower (~ 1nM) concentration of thrombin could be detected. Using even lower aptamer contruct concentrations (500 pM THR21 and 750 pM THR22), ~ 100 pM thrombin could be detected (not shown), but this low concentration of fluorescein-labeled aptamer construct is close to the limits of sensitivity of our instrumentation and the quality of the data was concomitantly decreased. To demonstrate the specificity of thrombin detection, we compared the response of the aptamer constructs to thrombin with the response to trypsin, a protease belonging to the same family as thrombin and sharing structural homologies with thrombin. No signal was detected upon addition of trypsin (Fig. 15B), indicating a high specificity of the aptamer constructs for thrombin.

[00154] Fig. 16 shows the results of competition experiments, in which the ability of various aptamer constructs to dissociate the preformed thrombin-aptamer construct complex was tested. The data obtained showed that all bivalent aptamer constructs were by far much more efficient competitors than any of the individual epitope-specific aptamers, in agreement with similar experiments performed with fluorescein-labeled individual aptamer (supra, THR2; Fig. 6). Among the bivalent aptamer constructs, THR18/THR19 (a construct with 27 nt long poly dT linker) and THR16/THR17 (a construct with 17 nt long poly dT linker) were the most efficient competitors followed by THR14/THR15 (a construct with 7 nt poly dT linker) and THR7 (which has a Spacer18 linker). It appears thus that although

additional flexibility of Spacer18 linkers was beneficial in terms of the magnitude of fluorescence signal change produced by the aptamer construct signal change, it also resulted in somewhat reduced affinity for binding thrombin in comparison with the constructs containing more rigid poly dT linkers.

Conclusions

[00155] We obtained data providing basic physicochemical characterization of the bivalent aptamer constructs containing two aptamers recognizing two different epitopes of thrombin. The bivalent constructs exhibited much higher affinity for thrombin than the individual aptamer components of the bivalent construct. This suggested that addition of thrombin to a mixture of aptamers "half-sites" should induce association of the two "half-sites" generating fluorescence signal as a result of bringing the fluorophore and the quencher to close proximity. Experiments with beacon constructs fully validated this prediction. We expect that it will be possible to develop analogous beacons for a large number of target proteins. We also note that the beacon design described here can also be adopted to improve beacons for detecting proteins exhibiting natural DNA binding activity (Fig. 1B). In this case one of the aptamers "half-sites" can be replaced with the DNA duplex (containing the protein binding site sequence) connected to signaling complementary oligonucleotide via flexible linker.

example 3: Analyte detection in a sample

Materials

[00156] Purified thrombin was a gift from Dr. Ray Rezaie (St. Louis University). Factor Xa, prothrombin, ovalbumin, bovine serum albumin, single-stranded binding protein, trypsin and plasma were purchased from Sigma (St. Louis, MO). HeLa cellular extracts were

from ProteinOne (College Park, MD). Texas Red-NHS and Sybr Green were from Molecular Probes (Eugene, OR), Cy5-NHS and Cy3-NHS were from Amersham Biosciences (Piscataway, NJ), and AMCA-sulfoNHS was from Pierce (Rockford, IL). All other reagents were commercially available analytical grade.

[00157] Oligonucleotide constructs used throughout this work are listed in Table 1. Oligonucleotides were obtained from Keck Oligonucleotide Synthesis Facility at Yale University or from IDT (Coralville, IA). 5' fluorescein and 3' dabcyl were incorporated using appropriate phosphoramidates during oligonucleoide synthesis. All other fluorophores were incorporated into oligonucleotides by post-synthetic modification of oligonucleotides containing 5' amino or C6 amino-dT at appropriate positions with NHS esters of the dyes. Oligonucleotides labeled with fluorescence probes were purified by reverse-phase HPLC as described previously (Heyduk, E.; Heyduk, T. *Anal. Biochem.* **1997**, 248, 216-227). Modification of oligonucleotides with europium chelate ((Eu^{3+})DTPA-AMCA) was performed by a two-step procedure described in Heyduk, E.; Heyduk, T.; Claus, P.; Wisniewski, J.R. *J. Biol. Chem.* **1997**, 272, 19763-19770. Concentrations of all oligonucleotides were calculated from UV absorbance at 260 nm after correction for the contribution of the fluorophore absorbance at 260 nm.

Fluorescence measurements

[00158] All fluorescence measurements were performed in 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$. Fluorescence spectra were recorded on Aminco Bowman Series 2 spectrofluorometer (Spectronic Instruments, Rochester, NY). Spectra were corrected for buffer and instrument response. Fluorescence in microplates was read with Tecan Spectra FluorPlus microplate reader (Research Triangle Park, NC). Alternatively,

microplates were imaged on Molecular Imager FX (BioRad, Hercules, CA) and fluorescence intensity was determined by integrating the areas of images corresponding to individual wells using QuantityOne software (BioRad). Experiments in 96-well plates and 384-well plates were conducted in 100 µl and 20 µl volume, respectively. Depending on particular instrumentation, slightly different beacon signal changes are recorded due to different buffer background readings with different instruments (depending on the sensitivity of the instrumentation) and different wavelengths of excitation and emission available with each instrument.

[00159] Time-resolved fluorescence in the case of europium chelate – Cy5 labeled beacons was recorded on a laboratory-built instrumentation (Heyduk, T.; Heyduk, E. *Analytical Biochemistry* **2001**, 289, 60-67), which employed pulsed nitrogen laser as the excitation source. Emission was integrated for 100 µs with 30 µsec delay after laser pulse.

Competition assay to determine thrombin aptamer dissociation constants.

[00160] Fluorescence intensity of THR2 in the presence and absence of the competitor was determined. Concentration of thrombin, THR2, and the competitor (when present) were 150 nM, 200 nM, and 200 nM, respectively. Under these conditions, binding of aptamers to thrombin was essentially stoichiometric. The previously described method (Matlock, D.L.; Heyduk, T. *Biochemistry* **2000**, 39, 12274-12283) was used to calculate the ratio of the dissociation constant for THR2 to that of the competitor under these experimental conditions.

Thrombin aptamer binding by electrophoretic mobility shift analysis (EMSA).

[00161] Five microliter samples of 417 nM THR7 were incubated with various amounts of thrombin (0 to 833 nM). After 15 min incubation, 1 μl of 30% Ficoll were added and the samples were run on a 10% polyacrylamide gel in TBE buffer. After the run the gel was stained for 30 min with Sybr Green and the image of the gel was obtained using Molecular Imager FX (BioRad). Intensity of the bands in the gel was determined by integrating the areas of image corresponding to individual bands using QuantityOne software (BioRad).

Design of aptamer-based molecular beacons

[00162] Fig. 3B illustrates the overall concept of molecular beacons for proteins lacking natural sequence-specific DNA binding activity. This design shares some general similarities with molecular beacons for DNA binding proteins described previously by inventor (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176; Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; Knoll, E.; Heyduk, T. *Analyt. Chem.* **2004**, 76, 1156-1164; Heyduk, E.; Fei, Y.; Heyduk, T. *Combinatorial Chemistry and High-throughput Screening* **2003**, 6, 183-194), (Fig. 3A). Instead of splitting the DNA duplex containing the natural binding site for a protein into the two "half-sites", two aptamers recognizing two no overlapping epitopes of the protein are used as functional equivalents of the "half-sites". Short complementary "signaling" oligonucleotides containing the fluorophore and the quencher are attached to the two aptamers via flexible linkers (Fig. 3B). In the absence of the target protein the two-aptamer "half-sites" can not associate since the complementary oligonucleotides are too short to promote efficient annealing. Binding of the

aptamer "half-sites" to the target protein brings the two "signaling" oligonucleotides into relative proximity increasing their local concentrations. This results in the annealing of the "signaling" oligonucleotides, which brings the fluorophore and the quencher into close proximity resulting in a change of fluorescence signal.

Properties of bivalent thrombin aptamers

[00163] We used thrombin as a model system to provide "proof-of-principle" verification of the concept illustrated in Fig. 3B. Thrombin is a proteolytic enzyme involved in the blood clotting cascade and naturally does not bind to DNA or RNA. Two laboratories have previously developed DNA aptamers, which selectively recognized two distinct epitopes of the protein (Bock, L.C.; Griffin, L.C.; Latham, J.A.; Vermass, E.H.; Toole, J.J. *Nature* **1992**, 355, 564-566, Tasset, D.M.; Kubik, M.F.; Steiner, W. *J. Mol. Biol.* **1997**, 272, 688-698). One aptamer (G15D; THR4, Table 1) was shown to bind to the heparin-binding exosite (Bock, 1992) whereas the other (60-18 [29]; THR3, Table 1) was shown to bind to fibrinogen-binding exosite (Tasset 1997). As a first step towards developing a beacon recognizing thrombin, we have prepared various aptamer constructs in which the above aptamers were covalently linked by flexible linkers. The primary purpose of these experiments was to determine if linking the two aptamers recognizing two distinct epitopes on a protein surface with a flexible linker would produce a bivalent aptamer capable of binding the protein with higher affinity compared to the individual aptamers. This is an essential condition for the assay illustrated in Fig. 3B to work. It was essential to experimentally address this question since it is impossible to predict the effect of long flexible linkers on the affinity of these bivalent constructs. A second purpose of these

experiments was to establish a suitable length of the linker and the appropriate orientation of 5' and 3' ends of the two aptamers with respect to the linker.

[00164] Individual aptamers were labeled with fluorescein (THR1 (Table 1), specific for fibrinogen-binding exosite, and THR2 (Table 1), specific for heparin-binding exosite) to facilitate determination of the affinity of various constructs for thrombin. Formation of a complex between thrombin and fluorescein-labeled 60-18 [29] aptamer (THR1) could be conveniently followed by fluorescence polarization (not shown) whereas binding of the fluorescein-labeled G15D aptamer (THR2) could be followed by changes in fluorescence intensity (Fig. 17A). Both aptamers bound thrombin in the nanomolar concentration range (data not shown for THR1 and Fig. 17A). Quantitative analysis of the binding in the case of THR2 (Fig. 17A) returned the value of K_d of 6.3 nM. This is somewhat higher affinity then previously suggested (Bock 1992, Tasset 1997) which is probably because we used a true equilibrium-binding assay whereas non-equilibrium methodology was used previously. When the binding of THR2 was performed in 10x excess of unlabeled 60-18 [29] aptamer (THR3) (Fig. 17B) only a small, insignificant decrease in affinity was observed (K_d was 17.7 nM). This confirmed that, as reported previously, G15D and 60-18 [29] aptamers bound independently to two distinct epitopes of thrombin.

[00165] In the next step, the ability of various aptamer constructs to compete with THR2 for binding to thrombin was evaluated. Fluorescence intensity change of THR2 upon addition of thrombin in the presence and absence of the competitor was measured and the amount of THR2 bound to thrombin in the presence of the competitor was calculated as described in Materials and Methods. No aptamer-aptamer interactions could be detected by fluorescence polarization assay (not shown) at aptamer concentrations used in these experiments indicating that the competition data correctly reported on the relative affinity of

THR2 and the competitor for binding to thrombin. THR3 was not a competitor (Fig. 17C) in agreement with the data shown in Fig. 17 A and B. THR4 (unlabeled variant of THR2), as expected, was able to compete (Fig. 17C). Quantitative analysis of the competition in this case showed that THR4 bound thrombin 1.7 times better then THR2 indicating that labeling this aptamer with fluorescein had small (insignificant) negative effect on aptamer binding to thrombin. It is obvious that all of the bivalent aptamer constructs were by far better competitors than THR4 (Fig. 17C). THR7 appeared to be the best competitor, essentially completely blocking THR2 binding at 1:1 ratio. Quantitative analysis of the competition in this case revealed that THR7 bound thrombin at least 65 fold tighter then THR2 (estimated K_d for THR7 was < 97 pM). The data shown in Fig. 17C confirmed the expectation that linking two aptamers recognizing two different epitopes of the protein with flexible linkers would produce high-affinity thrombin ligands. Additionally, these data showed that linking the two aptamers by a longer linker (containing 10 Spacer18 units vs. 5 Spacer18) produced slightly better affinity for thrombin (compare binding of THR5 vs. THR6). Also, these data showed that orientation of the aptamers with respect to the linker as in THR7 produced better affinity (compare affinity of THR6 vs. THR7). Thus, in all subsequent constructs, aptamer orientation as in THR7 was used.

[00166] The complex between the bivalent aptamer construct (THR7) and thrombin was stable enough to survive electrophoresis in native polyacrylamide gel (Fig. 17D). We took advantage of this observation and determined stoichiometry of the complex using electrophoretic mobility shift assay (EMSA) (Fried, M. G.; Crothers, D. M. *Nucleic Acid Res.* **1981**, 9, 6505-6525) to follow THR7-thrombin complex formation. We performed a titration of THR7 with thrombin at high concentrations of both molecules. Under these conditions the binding should be stoichiometric. The plot of the complex formed vs. the ratio

of thrombin to THR7 indicated 1:1 stoichiometry of the complex (Fig. 17D) consistent with the notion that both aptamers – components of THR7 – bind to their respective epitopes in THR7-thrombin complex.

Aptamer-based molecular beacon detecting thrombin

[00167] Experimental data described above provided evidence that all necessary conditions for successful implementation of the design of the signaling beacon shown in Fig. 3B were met. Based on these data we have designed the thrombin beacon illustrated in Fig. 13A. Thrombin aptamers were connected using 5 Spacer18 linkers to a 7 nucleotide ("nt") complementary oligonucleotides labeled at 5' and 3' with fluorescein and dabcyl, respectively. Mixture of these two constructs bound thrombin much more tightly (~ 36 times) compared to individual aptamers (Fig. 17C) in agreement with high affinity thrombin binding observed for bivalent aptamer constructs in which the two aptamers were permanently linked with a flexible linker. Addition of thrombin to a mixture of fluorochrome and quencher-labeled THR20 and THR21 resulted in protein concentration-dependent quenching of fluorescence intensity (Fig. 13C). Maximum quenching observed was ~ 40%. No fluorescence change was observed (Fig. 13C) upon addition of thrombin to THR21 in the absence of dabcyl-labeled partner (THR20) indicating that fluorescence quenching occurred due to protein-induced increased proximity of signaling oligonucleotides resulting in their annealing as illustrated in Fig. 13B. At nanomolar concentrations of the beacon components and thrombin ~ 15 min of incubation was sufficient to produce maximal response of the beacon. We have also tested thrombin beacons analogous to one shown in Fig. 13 but in which 17 nt poly dT linkers were used in place of Spacer18 linkers. While thrombin-dependent quenching of fluorescence was observed, the quenching was ~ 2 times smaller

than with the construct containing Spacer18 linkers. It is likely that poly dT linkers, while flexible, exhibited some residual rigidity (Mills, J.B.; Vacano, E.; Hagerman, P.J. *J. Mol. Biol.* **1999**, 285, 245-257), which perhaps might impede association of the signaling duplex when the two aptamers are bound to thrombin. When the beacon shown in Fig. 13 was titrated with trypsin, a proteolytic enzyme structurally similar to thrombin, no change of fluorescence intensity was observed. We concluded that a functional thrombin beacon according to the design illustrated in Fig. 3B was obtained.

Improvements in beacon performance

[00168] In the next set of experiments, we sought to improve the performance of the beacon by using alternative donor-acceptor label pairs. It has been shown previously that in assays employing FRET as the readout, enhancement of acceptor emission provides potentially better signal to background ratio, higher dynamic range, and better sensitivity (Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10). We have prepared a series of thrombin beacon constructs analogous to the one depicted in Fig. 3B, but in which various combinations of fluorescent donor and fluorescent acceptor were incorporated into the signaling oligonucleotides in place of fluorescein-dabcyl pair. THR21 (or THR28 labeled with appropriate NHS ester of the dye) and THR27 labeled with appropriate NHS ester of the dye were used to prepare these beacons. Fig. 18 shows fluorescence spectra of beacons (without and with thrombin addition) labeled with: fluorescein-Texas Red (Fig. 18B), fluorescein-Cy5 (Fig. 18C) and Cy3-Cy5 (Fig. 18D). In all cases functional beacons were obtained and with each beacon containing a fluorescent donor and fluorescent acceptor, a large thrombin concentration-dependent increase of sensitized acceptor emission was observed (Fig. 18, insets and Fig. 19 A-D). For comparison, Fig. 18A illustrates fluorescence

quenching observed in the presence of thrombin in the case of a fluorophore-quencher pair (fluorescein-dabcyl). Fig. 19E illustrates results obtained with europium chelate-Cy5 donor-acceptor pair which allowed the use of time-resolved FRET (TR-FRET) as a detection method (Selvin, P.R.; Rana, T.M.; Hearst, J.E. *J. Am. Chem. Soc.* **1994**, 116, 6029-6030; Selvin, P.R.; Hearst, J.E. *Proc. Natl. Acad. Sci USA* **1994**, 91, 10024-10028; Matthis, G. *Clinic. Chem.* **1995**, 41, 1391-1397). With TR-FRET it is possible to eliminate background due to light scattering and prompt fluorescence of directly excited acceptor to further improve signal-to-background ratio of the beacon. Fig. 19F summarizes the performance of beacon variants with various combinations of donor and acceptor probes. The figure shows the fold of signal change in the presence of saturating concentrations of thrombin compared to background signal of the beacon observed in the absence of the protein. This ratio varied from ~ 2 in the case of fluorescein-dabcyl pair to ~ 22 in the case of europium chelate-Cy5 pair. Thus, a substantial improvement of beacon performance can be obtained by selecting optimal donor-acceptor pairs and using sensitized acceptor emission as the mode of signal detection. An additional advantage of beacon variants with a fluorescent donor and a fluorescent acceptor is that their response can be measured by a two-color determination of the ratio of acceptor to donor signals. Such ratiometric measurement provides a more stable signal, which is more resistant to nonspecific effects due to light absorption, light scattering or fluorescence quenching caused by additives present in the sample. Increased signal-to-background ratio obtained with optimized donor-acceptor pairs resulted in an increased sensitivity of the beacon. This is illustrated in Fig. 20, which shows responses of three selected beacon variants to low concentrations of thrombin. In the case of the fluorescein-dabcyl labeled beacon (the lowest (~ 2 fold) signal change in the presence of saturating concentration of thrombin), statistically significant signal change could only be detected at

the highest thrombin concentration tested (1 nM). In the case of the fluorescein-Texas Red labeled beacon (~ 5 fold signal change at saturating thrombin concentration), statistically significant signal change could be detected at lower thrombin concentration (200 pM). In the case of the fluorescein-Cy5 labeled beacon (~ 15 fold signal change at saturating thrombin concentration), statistically significant signal change could be detected at the lowest thrombin concentration tested (50 pM).

[00169] Fig. 21illustrates the excellent reproducibility and stability of the thrombin beacon signal. The beacon signal was measured at four thrombin concentrations in five independent measurements. Coefficients of variation were small at each protein concentration tested (Fig. 21A). The beacon signal was stable for at least 24 hours (Fig. 21B).

[00170] Coincidence of three molecular contacts is required to generate a signal with the beacon illustrated in Fig. 3B: two contacts between each of the aptamers and the protein and the contact between the two complementary "signaling" oligonucleotides. Each of these contacts provides its own free energy contribution to the overall stability of beacon-protein complex. Due to an exponential relationship between the free energy and equilibrium dissociation constant of the complex, the overall stability of the complex would greatly decreased in the absence of any of the above three molecular contacts. Thus, it is expected that molecular beacons described here should exhibit greater specificity of protein detection compared to an assay based on a single molecular contact (for example, a single aptamer-based assay). To illustrate this concept we have compared the response of a single thrombin aptamer and thrombin beacon to SSB (Single Stranded DNA binding protein from *E. coli*), a protein exhibiting high nonspecific affinity for binding ss DNA (data not shown). SSB at nanomolar concentrations produced a large signal (as measured by fluorescence polarization assay) with the single, fluorescein-labeled aptamer (THR1, Table 1). SSB produced the

response in a concentration range very similar to the concentration of thrombin required to bind this aptamer. Thus, single thrombin aptamer exhibited very poor discrimination between SSB and thrombin. In contrast, exposure of thrombin beacon to nanomolar SSB concentration did not produce any significant beacon response, while thrombin at the same concentration range produces large beacon response. Thus, the thrombin beacon exhibited excellent discrimination between SSB and thrombin illustrating enhanced specificity of the beacon.

[00171] The primary application of the assay design described here will be in homogeneous high-throughput protein detection. Zhang et al. (*Biomol. Screening* **1999,** 4, 67-73) developed a simple statistical parameter, which could be used to evaluate assay for the use in a high-throughput manner. Z'-factor is calculated from large number of repeats of the measurement in the absence and the presence of the protein. Z' value of 1 indicates an ideal assay, Z' value of 0.5 to 1 indicates an excellent assay. Z' values below 0.5 indicate an assay not well suited for high-throughput applications. Z' value for the thrombin beacon was 0.94 (Fig. 22) which shows that it will be an outstanding high-throughput assay.

Detection of thrombin in complex mixtures

[00172] The next series of experiments addressed the specificity of the thrombin beacon and its ability to detect thrombin in cell extracts and in plasma. Response of the beacon to 1 nM thrombin was not affected by 100 and 1000 fold excess of unrelated protein (ovalbumin, Fig. 23A). Also, 100-fold excess of factor Xa, another clotting protease structurally similar to thrombin, did not affect the beacon response to 1 nM thrombin (Fig. 23A). A 1000-fold excess of factor Xa slightly attenuated the beacon response but 1 nM thrombin was still readily detectable under these conditions (Fig. 23A). Ovalbumin and factor

Xa up to 1 μM concentration had no effect on the beacon signal in the absence of thrombin (Fig. 23A). We concluded that the beacon was highly selective for thrombin.

[00173] To test if the beacon could detect thrombin in a complex mixture, we spiked HeLa cellular extract with varying amounts of thrombin and determined beacon response to this mixture (Fig. 23B). Low nanomolar concentrations of thrombin were readily detected. A total of 8 μg of protein were added to a 20 μl assay, which is within a typical range used in experiments with cellular extracts. The signal observed upon addition of cell extract could be completely abrogated by addition of a specific competitor (unlabeled thrombin aptamer) confirming that the observed signal in the cell extract was due to thrombin. One difficulty we've encountered working with cellular extracts was the degradation of oligonucleotides – components of the assay – by nucleases present in cellular extracts. We have tested various buffer additives to find conditions in which the thrombin beacon would remain stable in the presence of cell extracts for a sufficiently long period of time. We found that addition of high concentrations of random sequence 30 bp ds DNA (10 μM), high concentrations of 20 nt random sequence ss DNA (0.1μM), and 2.5 mM EGTA protected the thrombin beacon from degradation in the presence of cellular extracts without significantly affecting the response of the beacon to thrombin. Data shown in Fig. 23B were obtained in the presence of the above additives.

[00174] Since thrombin is a plasma protein, we determined if the beacon could be used to detect the protein in plasma. All of the thrombin in plasma is present in a precursor form, prothrombin, which is converted to thrombin via proteolytic processing by factor Xa. Prothrombin was recognized by the thrombin beacon albeit with much reduced (>20 fold) sensitivity compared to thrombin (not shown). This is well illustrated by the experiment

shown in Fig. 23C in which the sensitized acceptor emission of the beacon in the presence of prothrombin was monitored as a function of time. At the point marked by the arrow, factor Xa was added to the mixture to initiate conversion of prothrombin to thrombin. This conversion resulted in a time-dependent increase of beacon signal consistent with a much higher sensitivity of the beacon to thrombin. Thus, in order to detect thrombin in plasma, factor Xa was included in the assay mixture (Fig. 23D). Adding increasing amounts of plasma resulted in a proportional increase of beacon signal (Fig. 23D). Addition of plasma produced a response from the beacon only if factor Xa was present in the assay. The signal observed upon addition of plasma could be completely abrogated by addition of a specific competitor (unlabeled thrombin aptamer) confirming that the observed signal in the cell extract was due to thrombin. A 5 nL sample of plasma produced a measurable response of the beacon in a 20 μl reaction volume. In summary, the experiments illustrated in Fig. 23 demonstrated functionality of the thrombin beacon for detecting the protein in complex biological mixtures.

Discussion

[00175] The design of aptamer-based molecular beacons described here is a generalization of the design of molecular beacons for detecting sequence-specific DNA binding proteins previously developed by us (Fig. 3). Experiments with thrombin as a model protein presented here provide proof-of-principle evidence for the feasibility of this design. We believe this design will have several important advantages. Since the design of molecular beacons described here is not limited to any specific protein, it will be generally applicable to a large number of proteins. Signaling in the presence of the target protein by our beacon requires a cooperative recognition of two separate epitopes of the protein by two distinct

aptamers. This will result in an enhanced specificity of the beacon and increased affinity (i.e. sensitivity of detection). This cooperative action of two aptamers will also allow the use of aptamers with modest affinity to produce molecular beacons binding to target proteins with high affinity and specificity. Aptamers - components of the beacon, do not require any engineering of their structure to tailor their distribution of conformations to allow "switching" between different states in the presence of the protein. Such engineering could be dependent on a particular sequence (structure) of the aptamer and, such balancing of the energetics of alternative conformations of nucleic acids is not necessarily a trivial matter. Since the signaling elements ("signaling" oligonucleotides) in the instant beacon design are separate from its aptamer components, any aptamer sequence (and structure) should be compatible with our beacon design. It is also unlikely that the addition of the "signaling" oligonucleotides will have any deleterious effect on the affinity and specificity of the aptamer components of the beacon. Thus, any protein for which it will be possible to obtain two aptamers recognizing two distinct epitopes of the protein should be a good target for developing molecular beacons according to scheme in Fig.3.

[00176] Antibodies recognizing distinct epitopes of the protein can be obtained relatively easily. Similarly, there are no reasons why aptamers recognizing distinct epitopes could not be developed for many target proteins and several examples are already available (Jayasena, S.D. *Clinical Chem.* **1999**, 45, 1628-1650). Several approaches towards achieving this goal would be possible. The first approach would be to perform *in vitro* selections (SELEX) using different methods for separation of protein-bound and unbound oligonucleotides. The rationale here is that in these different partitioning methods different regions of the protein could be preferentially displayed resulting in aptamers directed to different regions of the protein surface. Aptamers selected to thrombin are an example of

such approach (Bock, 1992; Tasset, 1997). The second approach could be to raise the aptamers to peptides corresponding to different regions of the target protein molecule. Experimental evidence exists to show that such strategy can be used to develop aptamers capable of recognizing the intact protein from which the peptide used as a target for aptamer development was derived (Wei, X.; Ellington, A.D. *Proc. Natl. Acad. Sci. USA* **1996**, 93, 7475-7480). Such an approach is widely used to generate antibodies recognizing proteins. Two aptamers recognizing different epitopes of the protein can also be produced by a two-step sequential SELEX in which the second step involves selecting an aptamer in the presence of saturating concentration of the aptamer selected in the first step. We have validated this procedure using thrombin as a model system (Heyduk, E. and Heyduk, T., unpublished). Finally, we have developed a novel *in vitro* selection strategy to produce pairs of aptamers specifically designed to function in our molecular beacon design (Heyduk, E., Kalucka, J., Kinnear, B., Knoll, E., and Heyduk, T., unpublished). Thus, multiple routes to obtain pairs of aptamers recognizing non-overlapping epitopes of the protein will be available.

Example 4: Sensor Design Variations

[00177] Several variations of the instant molecular beacon are applicable in the practice of this invention. Those variants of the sensor design are depicted in Figure 24 and summarized herein (supra). The sensor design depicted in Fig. 24F is demonstrated to effectively detect DNA binding proteins. Upon the titration of cAMP response element binding protein ("CRP"), which is an example of a DNA binding protein, to a mixture of donor and acceptor labeled sensor components, there is a concomitant increase in sensitized acceptor fluorescence intensity (Figure 25B).

[00178] The sensor design depicted in Fig. 24G is demonstrated in Figure 26. Panel A depicts the principle of the sensor function. Upon the addition of single stranded DNA, which contains two distinct sequence elements that are complementary to elements in the sensor, to the mixture of two donor and acceptor labeled sensor components, there is a concomitant increase in sensitized acceptor fluorescence intensity (Figure 26, B, line with + sign). The sensor in this particular case contained Texas Red-labeled THR29 and THR32.

[00179] The increased specificity of the instant molecular beacon sensor design compared to assays based on a single, target macromolecule-recognizing element was experimentally demonstrated (Figure 27). Recognition of the target molecule by the sensor involves coincidence of three molecular contacts each providing a free energy (ΔG) contribution to the overall stability of the complex resulting in high specificity of target molecule recognition. Due to an exponential relationship between the free energy and equilibrium dissociation constant of the complex, the overall stability of the complex would greatly decrease in the absence of any of the above three molecular contacts. A nonspecific single stranded DNA binding protein ("SSB") at nanomolar concentrations produced a large signal (as measured by fluorescence polarization assay) with the single, fluorescein-labeled aptamer (THR1, Table 1). SSB produced the response in a concentration range very similar to the concentration of thrombin required to bind this aptamer. Thus, a single thrombin aptamer exhibited very poor discrimination between SSB and thrombin. (Panel B) Exposure of the thrombin sensor (a mixture of THR21 (fluorescein-labeled) and Texas Red labeled THR27) to nanomolar SSB concentration did not produce any significant beacon response (dashed lines), while thrombin at the same concentration range produces large beacon response (Panel C). Thus, the thrombin beacon exhibited excellent discrimination between SSB and thrombin, illustrating the enhanced specificity of the beacon.

Methods for preparing aptamers for the variant sensors

[00180] The selection of an aptamer binding to thrombin at an epitope distinct from the binding site of G15D aptamer was performed using the SELEX procedure starting from a construct containing a 33 nt random sequence (THR11) in the presence of excess G15D aptamer-containing construct (THR22) (Figure 28, panel A). Panel B depicts the thrombin binding activity of single stranded DNAs obtained after each indicated round of selection. Measurable thrombin binding activity appeared after the 4^{th} selection and reached a maximum after the 12^{th} selection. Binding was measured in the presence of the excess of THR22. DNA obtained after the12^{th} selection was cloned and DNA obtained from individual clones was sequenced. Panel C depicts the sequence alignment (using ClustalX) of the individual clones. Clones obtained from 4 independent selection experiments are shown. These selections were performed using the following pairs of aptamer constructs and random sequence-containing nucleic acid constructs: THR22 and THR 11; THR25 and THR 11; THR42 and THR11; THR43 and THR 11. Several families of highly conserved sequences are easily visible in panel C.

[00181] A functional thrombin sensor comprising Texas Red-labeled THR27 and fluorescein-labeled THR35 or THR36, which contain sequences corresponding to that of clones 20, 21, 24, and 26 from Fig. 28C, is depicted in Figure 29. THR35 and THR36 differ by the length of DNA sequence flanking the sequence of clones 20, 21, 24, and 26. The fluorescence image (sensitized acceptor emission) of wells of a microplate containing 20 nM (panel A) or 100 nM (panel B) of the indicated thrombin sensor and the indicated concentrations of thrombin are shown. For comparison, a sensor comprising THR21 and THR27 is shown.

[00182] Figure 30 depicts the results of simultaneously selecting two aptamers that bind to a target at distinct epitopes. The selection procedure began with two types of nucleic acid constructs, each containing a 30 nt random sequence (THR49 and THR50) (Table 1), and the target thrombin (panel A). Five mM of THR49 was added to 5mM THR50 in a total of 1 mL of buffer (50mM Tris-HCl (pH 7.5), 100mM NaCl, 5mM KCl and 1mM MgCl2). The mixture was boiled for approximately 1min, and allowed to cool to room temperature (RT). Thrombin (200nM) was added, and the mixture was incubated for 15-30 min at RT. Next, the mixture was spun through a nitrocellulose filter (NCF), followed by 2 washes of 1mL and a single wash of 0.5mL. Pre-warmed urea (200mL of 7M solution containing 1M NaCl) was loaded on the NCF, and incubated for 15min at 37°C. The DNA/urea mixture was eluted, and the DNA was precipitated with ethanol (added cold ethanol (2.5x the volume of eluted DNA) and incubated for at least two hours at –20°C). The precipitated DNA was centrifuged, the supernatant removed, and the subsequent pellet was dried in a speed-vac. The pellet was re-dissolved in 20mL of water, and used as a template for the PCR reaction.

[00183] Each PCR reaction contained 80mL of dd H2O, 10mL of 10xPCR buffer, 6mL of MgCl2, 0.8mL 25mM dNTPs, 1mL 50uM primer 1(modified with fluorescein), 1uL 50mM primer 2 (biotinylated), 0.5mL Taq polymerase, and 1mL of template. Two different sets of PCR reactions were performed corresponding to the two different types of nucleic acid constructs used (THR 49 and THR 50). The reaction cycle consisted of 5 min at 95°C, sixteen cycles of 30s at 95°C, 30s at 50°C, and 1min at 72°C, and 5min at 72°C. The samples were allowed to cool, and subsequently separated on a polyacrylamide gel. The band(s) of interest were visualized by utilizing the fluorescein tag, and were excised from the gel. The gel pieces were transferred to a microtube and crushed using a pipette tip. The gel pieces were covered with diffusion buffer (100mM Tris (pH 8.0), 0.5 M NaCl, 5mM EDTA) and the mixture was

incubated for at least two hours at 50°C. After centrifugation the supernatant was filtered through an empty Bio-Rad microspin column. The gel pieces were washed with fresh diffusion buffer, and the process repeated for a second time. The supernatants from the first and second procedures were combined.

[00184] Pre-equilibrated (1 M NaCl, 50mM Tris (pH 8.0), and 1 mM EDTA) DYNAL magnetic streptavidin beads were mixed with the gel-purified DNA, and incubated at RT for 30min with constant shaking. The supernatant was removed, and the beads were washed once with 500mL, once with 250mL, and once with 100mL of buffer. Next, the beads were incubated for 30min at 37°C with 50mL of 0.15N NaOH. The supernatant containing the fluorescein labeled DNA was removed and filtered through a G-25 Sephadex microspin column pre-equilibrated with buffer. The estimated concentration of the recovered DNA was calculated by comparison to a known amount of fluorescein-labeled primer.

[00185] The second round of selection began by combining 50nM of the recovered DNA and 50-1000nM of THR22 in a total of 50mL of selection buffer. The DNA mixture was boiled for 1min, and allowed to cool to RT. Subsequently, the DNA mixture was filtered through a pre-equilibrated NCF to remove DNA sequences with affinity for the NCF. Thrombin (20nM) was added to the filtered DNA and the mixture was incubated for 15-10 min at RT. Next, the mixture was spun through another pre-equilibrated NCF, followed by two washes of 100mL. After incubation with 100mL of urea (7M in a buffer of 1M NaCL) for 15min at 37°C, the DNA-thrombin complexes were eluted from the NCF. The DNA in the eluted solution was precipitated with alcohol (see above) and re-suspended in 20mL of water. This was used as a template for the PCR reaction. PCR products were purified by electrophoresis on a polyacrylamide gel and the single-stranded DNA was obtained from

purified PCR products as described above for the first selection. Subsequent selections were repeated until the detected thrombin-binding activity reached a maximum (Figure 30B).

[00186] The thrombin-binding activity of the mixture of single-stranded DNAs obtained after each indicated round of selection is shown in panel B. Measurable thrombin-binding activity appeared after the 8th selection and reached a maximum after the 13th selection. DNA obtained after the 13th selection was cloned and the DNA from individual clones was sequenced. Panel C depicts the sequence alignment (using ClustalX) of the clones. Several families of highly conserved sequences are easily visible.

[00187] Aptamer-based molecular beacons were developed for cAMP response element binding protein ("CRP"). Aptamers were selected to bind at sites distinct from the DNA binding site of the protein. Selection was performed using the SELEX procedure starting from a construct containing a 33 nucleotide random sequence (MIS12) in the presence of excess of CRP binding site-containing construct (MIS10X3 hybridized with MIS11) (Figure 31, panel A). CRP binding activity of single stranded DNA that was obtained after indicated round of selection is depicted in Figure 31, panel B. Measurable CRP binding activity appeared after 6^{th} selection and reached maximum after 12^{th} selection. Binding was measured in the presence of excess MIS10X3 hybridized with MIS11. DNA obtained after 12^{th} selection was cloned and DNA obtained from the individual clones were sequenced. The sequence alignment (using ClustalX) of the clones is depicted in panel C. Conserved core sequence of ~16 nucleotides could be identified.

Table 1

CONSTRUCT	*SEQUENCE*	*SEQUENCE IDENTIFIER*	*DESCRIPTION*
THR1	5' Fluorescein AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:1	60-18 [29][a] aptamer labeled with fluorescein
THR2	5' Fluorescein GGT TGG TGT GGT TGG	SEQ ID NO:2	G15D[b] aptamer labeled with fluorescein
THR3	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:3	60-18 [29] aptamer
THR4	GGT TGG TGT GGT TGG	SEQ ID NO:4	G15D aptamer
THR5	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT $(Spacer18)_5$ GG TTG GTG TGG TTG G	SEQ ID NO:5	60-18 [29] aptamer connected to G15D aptamer via 5 Spacer18 linkers
THR6	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT $(Spacer18)_{10}$ GGT TGG TGT GGT TGG	SEQ ID NO:6	60-18 [29] aptamer connected to G15D aptamer via 10 Spacer18 linkers
THR7	GGT TGG TGT GGT TGG $(Spacer18)_{10}$ AG TCC GTG GTA GGG CAG GTT GGG GTG ACT	SEQ ID NO:7	G15D aptamer connected to 60-18 [29] aptamer via 10 Spacer18 linkers
THR20	GGT TGG TGT GGT TGG $(Spacer18)_5$ C GCA TCT 3'dabcyl	SEQ ID NO:16	G15D aptamer connected via 5 Spacer18 linkers to 7 nt "signaling" oligonucleotide labeled with dabcyl at 3' end
THR21	5' fluorescein AGA TGC G $(Spacer18)_5$ AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:17	7 nt "signaling" oligonucleotide labeled at 5' with fluorescein connected to 60-18 [29] aptamer via 5 Spacer18 linkers

THR27	GGT TGG TGT GGT TGG (Spacer18)$_5$ (C6 amino-dT) C GCA TCT	SEQ ID NO:18	G15D aptamer connected via 5 Spacer18 linkers to 7 nt "signaling" oligonucleotide containing amino-dT (near its 5' end)
THR28	5' amino AGA TGC G (Spacer18)$_5$ AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:19	7 nt "signaling" oligonucleotide containing 5' amino connected to 60-18 [29] aptamer via 5 Spacer18 linkers
THR11	CTG TCG TTA GTG AAG GTT NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN AAC GCC ATA TCA CAG ACG	SEQ ID NO:20	Construct containing 33 nt random DNA sequence for thrombin aptamer selection
THR12	5' fluorescein CTG TCG TTA GTG AAG GTT	SEQ ID NO:21	Primer1 for THR11
THR13	5' biotin CGT CTG TGA TAT GGC GTT	SEQ ID NO:22	Primer2 for THR11
THR22	GGT TGG TGT GGT TGG (Spacer18)$_2$ GA CAG	SEQ ID NO:23	Co-aptamer for thrombin aptamer selection
THR25	GGT TGG TGT GGT TGG (Spacer18)$_5$ AC GA CAG	SEQ ID NO:24	Co-aptamer for thrombin aptamer selection
THR29	GAACGAGAGTGC XXXXX amino C GCA TCT	SEQ ID NO:25	ss DNA sensor component
THR32	5' fluorescein AGA TGC G XXXXX TTGAACTGGACC	SEQ ID NO:26	ss DNA sensor component
THR33	GGTCCAGTTCAA TT GCACTCTCGTTC	SEQ ID NO:27	target ss DNA for ss DNA sensor
THR42	GGT TGG TGT GGT TGG XX XXX AAC GAC AG	SEQ ID NO:28	co-aptamer for thrombin aptamer selection

THR43	CTG TCG TT $(Spacer18)_5$ TTGAGTCAGCGTCGAG CA NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN TTC ACT GTG CTG CGG CTA	SEQ ID NO:29	Construct containing 33 nt random DNA sequence for thrombin aptamer selection
THR44	5' fluorescein CTG TCG TT $(Spacer18)_5$ TTG AGT CAG CGT CGA GCA	SEQ ID NO:30	Primer1 for THR43
THR45	5'biotin TAGCCGCAGCACAGTG AA	SEQ ID NO:31	Primer2 for THR43
THR49	CACCTGATCGCTCCTCG T NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CAG GAT GCA CAG GCA CAA	SEQ ID NO:32	Construct containing 30 nt random DNA sequence for simultaneous selection of two thrombin aptamers
THR50	AGCCGCCATTCCATAGT G NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CAG GAT GCC GAT CAG GTG	SEQ ID NO:33	Construct containing 30 nt random DNA sequence for simultaneous selection of two thrombin aptamers
THR51	5' fluorescein CAC CTG ATC GCT CCT CGT	SEQ ID NO:34	Primer1 for THR49
THR52	5'biotin TTG TGC CTG TGC ATC CTG	SEQ ID NO:35	Primer2 for THR49
THR53	5' fluorescein-AGC CGC CAT TCC ATA GTG	SEQ ID NO:36	Primer3 for THR50
THR54	5'biotin CAC CTG ATC GGC ATC CTG	SEQ ID NO:37	Primer4 for THR50
THR35	5' fluorescein AGA TGC G $(Spacer18)_5$ AG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AAC GC	SEQ ID NO:38	Thrombin sensor component
THR36	5' fluorescein AGA TGC G $(Spacer18)_5$ A GTG AAG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AAC GCC ATA T	SEQ ID NO:39	Thrombin sensor component

MIS10X3	AAC GCA ATA AAT GTG AAG TAG ATC ACA TTT TAG GCA CC (Spacer18)$_5$ GA TGGCT	SEQ ID NO:40	co-aptamer for CRP aptamer selection
MIS12	AGCCA T CTA ACT ATT CCC NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN GAG CGA GAA ATT CTA GGT	SEQ ID NO:41	Construct containing 33 nt random DNA sequence for CRP aptamer selection
MIS11	GGT GCC TAA AAT GTG ATC TAC TTC ACA TTT ATT GCG TT	SEQ ID NO:42	Complement to MIS10X3
MIS13	5'-fluorescein - AGC CA T CTA ACT ATT CCC	SEQ ID NO:43	Primer1 for MIS10X3
MIS14	5' biotin- ACC TAG AAT TTC TCG CTC	SEQ ID NO:44	Primer2 for MIS10X3
Clone 1	ggcggtatgg gcatagcgta atgggaggtt ggt	SEQ ID NO:45	Clone from Figure 28
Clone 2	ggatgcgtaa tggttagggt gggtagggta tcc	SEQ ID NO:46	Clone from Figure 28
Clone 3	ggatgcgtaa tggttagggt gggtagggta tcc	SEQ ID NO:47	Clone from Figure 28
Clone 4	ggatgcgtaa tggttagggt gggtagggta tcc	SEQ ID NO:48	Clone from Figure 28
Clone 5	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:49	Clone from Figure 28
Clone 6	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:50	Clones from Figure 28
Clone 7	ggcggtatgg gcatagcgta atgggaggtc tgc	SEQ ID NO:51	Clone from Figure 28
Clone 8	ggatgcgtaa tggttagggt gggtagggta tcc	SEQ ID NO:52	Clone from Figure 28
Clone 9	ggcggtatgg gtatagcgta atgggaggtt ggt	SEQ ID NO:53	Clone from Figure 28
Clone 10	gggggtacta ggtattaatg ggtagggtgg tgt	SEQ ID NO:54	Clone from Figure 28
Clone 11	cagcagggaa cggaacggtt agggtgggta ggg	SEQ ID NO:55	Clone from Figure 28
Clone 12	gcggngatag gtcgcgtaag ttgggtaggg tgg	SEQ ID NO:56	Clone from Figure 28
Clone 13	caggatgggt agggtggtca gcgaagcagt agg	SEQ ID NO:57	Clone from Figure 28
Clone 14	caacggttgg gtgaactgta gtggcttggg gtg	SEQ ID NO:58	Clone from Figure 28
Clone 15	caggatgggt agggtggtca gcgaagcagt agg	SEQ ID NO:59	Clone from Figure 28

Clone 16	caggatgggt agggtggtca gcgaagcagt ag	SEQ ID NO:60	Clone from Figure 28
Clone 17	ggcgagagca gcgtgatagg gtgggtaggg tgg	SEQ ID NO:61	Clone from Figure 28
Clone 18	cagggtcagg gctagatgat gcgattaacc atg	SEQ ID NO:62	Clone from Figure 28
Clone 19	caggatgggt agggtggtca gcgaagcagt agg	SEQ ID NO:63	Clone from Figure 28
Clone 20	gggggtacta ggtatcaatg ggtagggtgg tgt	SEQ ID NO:64	Clone from Figure 28
Clone 21	gggggtacta ggtatcaatg ggtagggtgg tgt	SEQ ID NO:65	Clone from Figure 28
Clone 22	ggagacgtaa tgggttggtt gggaagngga tcc	SEQ ID NO:66	Clone from Figure 28
Clone 23	gcatacgtaa tggtccggtt ggggcgggta tgt	SEQ ID NO:67	Clone from Figure 28
Clone 24	gggggtacta ggtatcaatg ggtagggtgg tgt	SEQ ID NO:68	Clone from Figure 28
Clone 25	gaggggactt aggatgggta gggtggtagg ccc	SEQ ID NO:69	Clone from Figure 28
Clone 26	gggggtacta ggtatcaatg ggtagggtgg tgt	SEQ ID NO:70	Clone from Figure 28
Clone 27	ggtcggggca tagtaatgct ggattgggca gct	SEQ ID NO:71	Clone from Figure 28
Clone 28	gggtaggagc agtacacgct ggaatgggtc act	SEQ ID NO:72	Clone from Figure 28
Clone 29	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:73	Clone from Figure 28
Clone 30	gggtagggtg acagggagga cggaatgggc act	SEQ ID NO:74	Clone from Figure 28
Clone 31	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:75	Clone from Figure 28
Clone 32	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:76	Clone from Figure 28
Clone 33	gcagtaggta ctatattggc tagggtggtc tgc	SEQ ID NO:77	Clone from Figure 28
Clone 34	gggggtgcta ggtattaaag ggtagggtgg tgt	SEQ ID NO:78	Clone from Figure 28
Clone 35	gcagtaggta ctatgtcggg tcgggtggtc tgc	SEQ ID NO:79	Clone from Figure 28
Clone 1-1	gggtagggtg gttgtaatag ggattgcgat	SEQ ID NO:80	Clone from Figure 30
Clone 1-2	gggtagggtg gttgtaatag ggattgcgat	SEQ ID NO:81	Clone from Figure 30
Clone 1-3	ggcacaaccc gatatggcta tgaatctgcc	SEQ ID NO:82	Clone from Figure 30

Clone 1-4	gggtagggtg gttgtaatag ggattgcgat	SEQ ID NO:83	Clone from Figure 30
Clone 1-5	gggtagggtg gttgtaatag ggattgcgat	SEQ ID NO:84	Clone from Figure 30
Clone 1-6	ggtgtgggtg gttattggtg tagagcgggt	SEQ ID NO:85	Clone from Figure 30
Clone 1-7	aatggggagg ttggggtgcg ggagagtggt	SEQ ID NO:86	Clone from Figure 30
Clone 1-8	acgcgtagga tgggtagggt ggtcgcgtta	SEQ ID NO:87	Clone from Figure 30
Clone 1-9	gggtagggtg gttgtaatag ggattgcgat	SEQ ID NO:88	Clone from Figure 30
Clone 1-10	gggcgaaggt acgaagacgg atgcacgtgc	SEQ ID NO:89	Clone from Figure 30
Clone 2-1	aaggccgcca tctgggtccg acgagtacca	SEQ ID NO:90	Clone from Figure 30
Clone 2-2	tagggtgggt agggtggtca actatggggg	SEQ ID NO:91	Clone from Figure 30
Clone 2-3	gggtggctgg tcaaggagat agtacgatgc	SEQ ID NO:92	Clone from Figure 30
Clone 2-4	ggtagggtgg ttaaaatagg ggaatggcag	SEQ ID NO:93	Clone from Figure 30
Clone 2-5	cacaagaagg gcgagcgctg agcatagtgc	SEQ ID NO:94	Clone from Figure 30
Clone 2-6	ccaacgacac atagggtaca cgccgcctcc	SEQ ID NO:95	Clone from Figure 30
Clone 2-7	ggtagggtgg ttaaaatagg ggaatggcag	SEQ ID NO:96	Clone from Figure 30
Clone 2-8	taggatgggt agggtggtcc caggaatggc	SEQ ID NO:97	Clone from Figure 30
Clone 2-9	taggatgggt agggtggccc caggaatggc	SEQ ID NO:98	Clone from Figure 30
Clone 2-10	ggtagggtgg ttaaaatagg ggaatggcag	SEQ ID NO:99	Clone from Figure 30
Clone 2-11	gatgtggccc agaagcataa cacgacgtac	SEQ ID NO:100	Clone from Figure 30
Clone 2-12	taggatgggt agggtggtcc caggaatggc	SEQ ID NO:101	Clone from Figure 30
Clone 2-13	ggagatgcag gtactgagta gggagtgtgc	SEQ ID NO:102	Clone from Figure 30
Clone 2-14	taggatgggt agggtggtcc caggaatggc	SEQ ID NO:103	Clone from Figure 30
Clone 1	aatcaagggc tggtgttaaa ggtgatcgac tag	SEQ ID NO:104	Clone from Figure 31
Clone 2	aaggggagcc atcacacagg aggtcgcttc gct	SEQ ID NO:105	Clone from Figure 31

Clone 3	aaaggcatca cctagagttg ccgccgatac ttg	SEQ ID NO:106	Clone from Figure 31
Clone 4	ggggatgtgc gaaactggtg actatgcggg tgc	SEQ ID NO:107	Clone from Figure 31
Clone 5	cgaaaggagc catcaacctt gaaacgcccg tcc	SEQ ID NO:108	Clone from Figure 31
Clone 6	cagacgggag ccatcgacat agaggtgatt gcc	SEQ ID NO:109	Clone from Figure 31
Clone 7	agggaaagcc atcacctaga cacatacagc atg	SEQ ID NO:110	Clone from Figure 31
Clone 8	ataagaagcc atcataggga cctagctagc ccc	SEQ ID NO:111	Clone from Figure 31
Clone 9	ccaacagacg gtagcacaac actagtactc tgg	SEQ ID NO:112	Clone from Figure 31
Clone 10	acagacgccc ctagtaaaca ataaccgatg gcc	SEQ ID NO:113	Clone from Figure 31
Clone 11	atagctactc gccaagggtg acttctgcta ttg	SEQ ID NO:114	Clone from Figure 31
Clone 12	atggggcaac gcggagacct gtcggtactg cct	SEQ ID NO:115	Clone from Figure 31
Clone 13	gcaatatagc actaagcctt aactccatgg tgg	SEQ ID NO:116	Clone from Figure 31
Clone 14	gcaaggaaaa acaagcaagc catcacgacc tag	SEQ ID NO:117	Clone from Figure 31
Clone 15	caggcatccc aagaagtgtc agccgtttcg tgg	SEQ ID NO:118	Clone from Figure 31
Clone 16	caacaggaga gcccgacaca cagatctggc ccc	SEQ ID NO:119	Clone from Figure 31
Clone 17	acaagccatc acgtgaatgc cgaccggtac tgt	SEQ ID NO:120	Clone from Figure 31
Clone 18	accgacaaac aagtcaatac gggacacgat cct	SEQ ID NO:121	Clone from Figure 31
Clone 19	cagtgggtcg ggtcacagcc atgagtgttg ctg	SEQ ID NO:122	Clone from Figure 31
Clone 20	aacgggaaag ccatcaccat atttatcgtc ctg	SEQ ID NO:123	Clone from Figure 31
Clone 21	acgggcgcaa acaagatgta caaaagcatg gtg	SEQ ID NO:124	Clone from Figure 31
Clone 22	agcgggatag ggaactatcg gacaatcgtc gtg	SEQ ID NO:125	Clone from Figure 31
Clone 23	gaggataaaa gccatcaact agaatgcgca tgg	SEQ ID NO:126	Clone from Figure 31